EFFECTS OF A TERATOGEN ON YOLK-SAC FUNCTION

By

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DEDICATION

To my parents for their unparalleled encouragement and concern and to my wife for her generous support, this dissertation is lovingly dedicated.

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INTRODUCTION

The rat embryo, unlike most mammals, undergoes the last two-thirds of gestation and its true morphogenetic sequences enclosed within the yolk-sac membrane (Fig. 1). The yolk-sac, which is of endodermal origin, is composed of a proximal and a distal portion. The proximal (visceral) membrane (Fig. 2A) is the more complex of the two and separates the extraembryonic celom initially from the yolk-sac cavity and, after rupture of the distal (parietal) component on day 14 of gestation, from the uterine lumen.

Although the function of the yolk-sac and its role in embryonic and fetal differentiation are essentially unknown, studies of yolk-sac physiology and morphology have indicated that the proximal yolk-sac may function in the transfer of material to or from the developing embryo and growing fetus. The present study was therefore designed to first elucidate a possible absorptive function for the visceral yolk-sac and second, to determine the effect of a potent teratogenic agent on the concentrating ability of the yolk-sac.

Morphology of the Rat's Yolk-Sac

Parietal Yolk-Sac

The parietal yolk-sac is not present as a complete surrounding membrane throughout gestation (21 days), since it ruptures during day 14[1]

[1]All days of gestation have been modified to correspond to that described in Materials and Methods.

Fig. 1.—Schematic drawing of a typical rat implantation site. Abbreviations:

cap = chorio-allantoic placenta
cav = chorio-allantoic vessels
emb = embryo
ex = exocelom
ysc = yolk-sac cavity
vys = visceral yolk-sac
A = see legend for Figure 2A
end = endodermal sinus
vi = villi
vv = vitelline vessels
am = amnion
ul = uterine lumen
pys = parietal yolk-sac
B = see legend for Figure 2B

Fig. 2.—Schematic drawings demonstrating the microscopic structure of the visceral yolk-sac and the parietal yolk-sac.

A.—The visceral yolk-sac. Abbreviations:

mth = mesothelium
mes = mesenchyme
vbm = visceral basement membrane
pv = pinocytotic vesicles
mv = microvilli
ysc = yolk-sac cavity
sbm = serosal basement membrane
vv = vitelline vessel
fp = foot process
ac = apical canaliculi
ve = visceral endoderm

B.—The parietal yolk-sac. Abbreviations:

pe = parietal endoderm
ml = maternal labyrinth
rm = Reichert's membrane
gc = giant cell

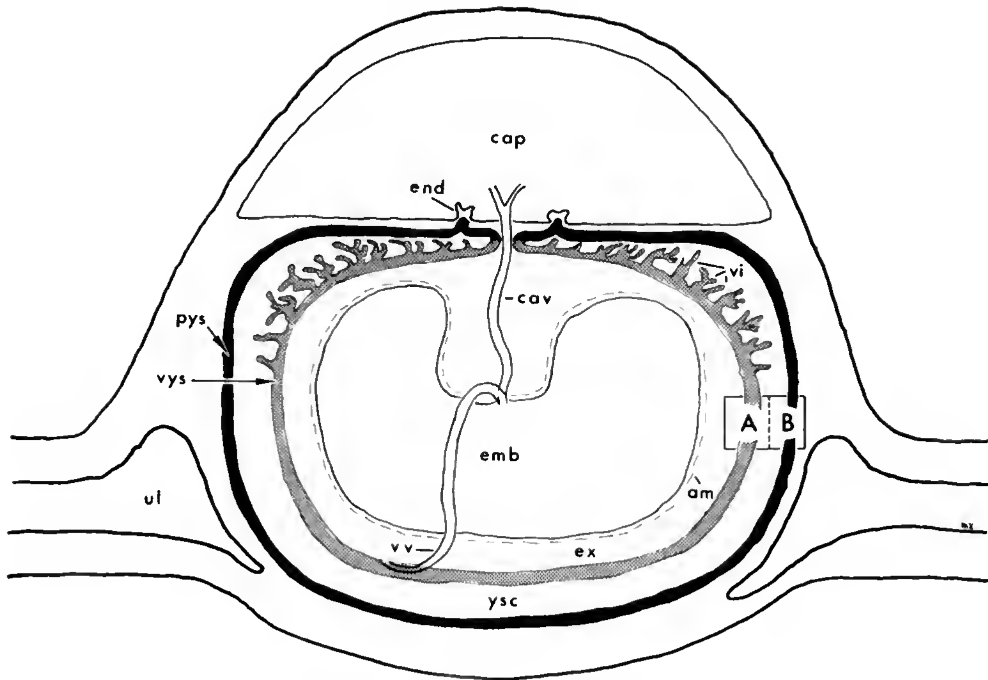

Fig. 1

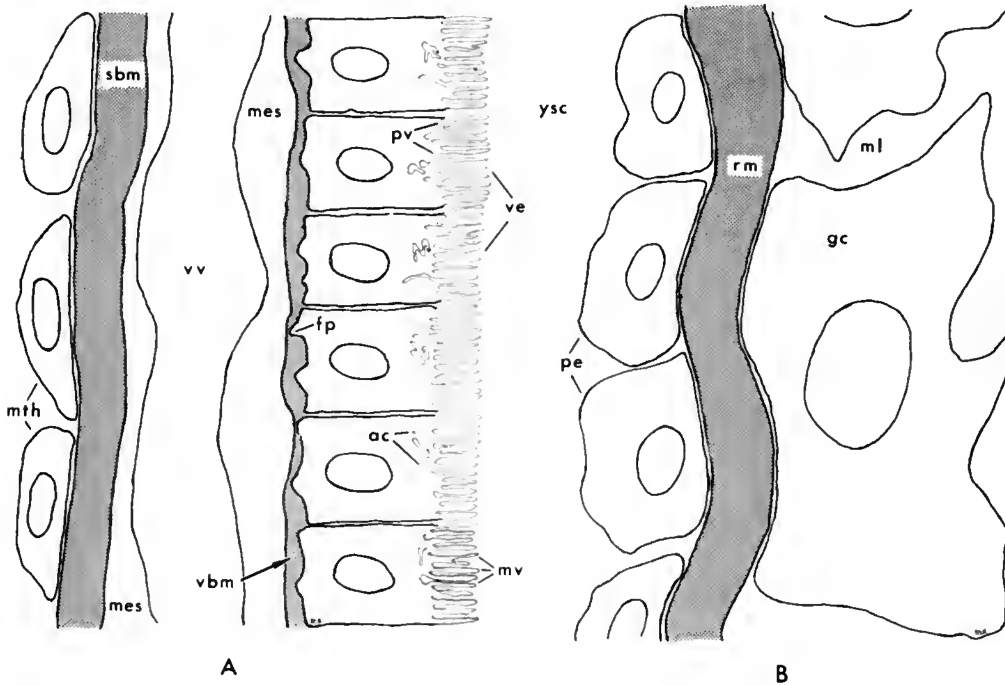

Fig. 2

of gestation resulting in loss of the antimesometrial portion. Remnants of Reichert's membrane then recoil to the perimeter of the chorio-allantoic placenta, thereby causing the yolk-sac cavity to become confluent with the reforming uterine lumen (Wislocki and Padykula, 1953; Padykula and Richardson, 1963).

Prior to its rupture, the distal yolk-sac consists of three layers (Fig. 2B). The layer on the maternal side of the distal yolk-sac is composed of the so-called giant cells or "central zone" (Everett, 1935). These are large, spindle-shaped cells of unknown derivation separated by intercommunicating spaces (the labyrinth), through which flows maternal blood. This blood is completely replaced every 20 minutes (Everett, 1935) contrary to previously published reports by Sobotta (1903), Asai (1914) and Grosser (1927), who believed it to be an immobile pool, gradually incorporated into the embryo. The embryonic side of the distal yolk-sac consists of small, noncontiguous parietal endoderm cells which seem capable of ameboid movement and phagocytosis (Everett, 1935; Gérard, 1925). Between these two ill-defined layers is an obvious basement (Reichert's) membrane which is loosely connected to the innermost of the giant cells and has been variously considered as an ectodermal cuticle (Duval, 1892), a protoplasmic membrane (Sobotta, 1903), a basement membrane (Grosser, 1927) and a hyaline membrane (Mossman, 1937). More recent observations (Wislocki and Padykula, 1953) have suggested that Reichert's membrane is composed of fibers very similar to compact collagenous fibers. On the basis of certain histochemical reactions, Reichert's membrane is similar to the lens capsule and Descement's membrane in the cornea.

Ultrastructurally, Reichert's membrane is characterized by wavy bundles of fibrils unlike other basement membranes. Wislocki and Dempsey (1955) have suggested that the membrane is composed of a considerable amount of ground substance which masks the usual visualization of collagen with the electron microscope.

Visceral Yolk-Sac

The visceral wall of the yolk-sac is composed of three cellular layers separated by two basement membranes (Fig. 2A). The outer layer is the visceral endoderm or vitelline epithelium consisting of columnar cells over the mesometrial one-half and low cuboidal cells over the antimesometrial hemisphere (Everett, 1935).

By day 12 of gestation, the free or apical surfaces of the cells of the vitelline epithelium are evaginated into a microvillous border. In the ensuing two days, the length and density of the microvilli increase and reach their maximum development at day 14. From day 15 to term, they recede but do not disappear. Between the microvilli and projecting for variable distances into the apical cytoplasm are a series of indentations (the apical canaliculi) which vary from small pinocytotic invaginations to a series of interconnecting tubules. These canaliculi are well-developed from day 12 until term, but, later in gestation, they become increasingly dilated (Padykula, et al., 1966).

The microvilli are invested with a filamentous glycoprotein coat (the glycocalyx; Bennett, 1963) which also seems to line all invaginated canaliculi as well as the inner surfaces of some of the intracellular vacuoles. Both colloidal gold (Luse, 1958) and ferritin

(Lambson, 1966) adhere to the glycocalyx on the surfaces of the cells as well as within the canaliculi and vacuoles.

The lateral intercellular relationships also change during the course of gestation. At days 12 and 13, the lateral cell membranes of adjacent cells appear tightly sealed from the apical surface to about two-thirds down the length of the cell where they are thrown into complex folds and interdigitations. Near term, however, only the most apical portions of the cells appear to be tightly bound together and below this, large dilatations containing a finely granular material appear. Only occasionally do the membranes of two adjacent cells meet to form a desmosome. Excluding small foot processes which extend into the underlying basement membrane, the basal cell membranes have no remarkable specializations or sequential differentiation.

The middle cellular layer of the proximal yolk-sac, the mesoderm, becomes vascularized by the irregular vitelline blood vessels beginning on day 10. The inner cellular layer is a mesothelium composed of cells unusually rich in endoplasmic reticulum (Wislocki and Dempsey, 1955).

Beneath the visceral endoderm is a narrow visceral basement membrane which thickens as gestation continues. In contrast, the serosal basement membrane, between the mesenchymal and mesothelial layers, is stout and appears to be rich in collagen.

Villi begin to form in the visceral yolk-sac (Fig. 1) during the tenth day of gestation. The villi become taller and more branched as gestation continues (Everett, 1935).

The proximal yolk-sac immediately surrounding the exit of the chorio-allantoic blood vessels is covered by low cuboidal to squamous cells. This area of the proximal yolk-sac is avascular.

Endodermal Sinuses

The endodermal sinuses are invaginations of the yolk-sac cavity into the chorio-allantoic placenta (Fig. 1). The placental surfaces of the sinuses are lined by Reichert's membrane and distal endodermal cells, both of which appear continuous with the parietal yolk-sac. The opposite sides of the sinuses are lined by cuboidal cells which, though continuous with the vitelline epithelium, differ in cytological properties (Wislocki and Padykula, 1953). These cells are small and cuboidal and contain no stores of glycogen. They demonstrate no brush border, no well-defined basement membrane and rest on a stroma of allantois rather than splanchnopleure.

Chemical Constitution of the Visceral Yolk-Sac

Wislocki and Padykula (1953) employed histochemical techniques to demonstrate the presence of glycogen, glycoproteins, mucopolysaccharides and lipids within the cytoplasm of the vitelline epithelium. These cells exhibit alterations in chemical concentrations and composition with increasing gestational age. For example, the visceral yolk-sac initiates glycogen storage in both the vitelline epithelium and mesenchymal layer by day 13. The stores increase through day 18, and then decrease to term.

Yolk-sacs explanted into a culture medium also have the ability to store glycogen (Sorokin and Padykula, 1964), although in a different temporal sequence. Under these conditions, glycogen accumulation begins at day 13, reaches and maintains a maximum level at days 20 to 25 of incubation and then declines. These results suggest two critical hypotheses. First, the yolk-sac does not rely upon maternal or embryonic

influences to initiate or maintain glycogen storage. Second, the *in vivo* decrease in glycogen concentration is apparently due to a stimulus in the form of a decrease of substrate, change in the hormonal environment or other such environmental factor and is not the result of age or senescence of the yolk-sac itself. Whether the glycogen is eventually transferred to the embryo or is utilized as a substrate for yolk-sac metabolism or is utilized elsewhere are questions which are, as yet, unanswered.

Some of the enzyme constituents of the visceral yolk-sac also have been characterized (Padykula, 1958). The enzymes studied were succinic dehydrogenase, nonspecific esterases, acid phosphatase, alkaline phosphatase and adenosine triphosphatase. In general, all enzymatic activity was low at day 12, increased by day 14, reached a peak on days 15 (succinic dehydrogenase), 16 (alkaline phosphatase) or 18 (adenosine triphosphatase) and thereafter declined. In addition, enzymatic activity was localized over certain areas of the cell. For example, alkaline phosphatase activity was most intense in the apical cytoplasm and brush border, acid phosphatase and adenosine triphosphatase in the supranuclear cytoplasm and succinic dehydrogenase in the basal cytoplasm.

General alterations in enzymatic activity during the ontogeny of the yolk-sac recently have been demonstrated by Johnson and Spinuzzi (1966). These investigators used electrophoresis to study the effects of a teratogenic agent on the differentiation of various molecular forms of enzymes in the yolk-sac. Other enzymes which have been

identified in the visceral yolk-sac of the rat are malate and lactate dehydrogenases (Johnson and Spinuzzi, 1966), beta-glucuronidase (Bulmer, 1963; Beck et al., 1967) and ribonuclease and deoxyribonuclease (Beck et al., 1967).

The visceral basement membrane of the proximal yolk-sac was shown also to undergo a histochemical differentiation (Wislocki and Padykula, 1953). At day 10 of gestation, the membrane is so thin that it is barely perceptible with the light microscope but as gestation continues, it becomes increasingly thicker. At the height of its development, it consists of reticular fibers, collagen and periodic acid-Schiff (PAS)-positive ground substance. In these respects, the visceral basement membrane is characteristic of other such membranes located throughout the maternal and fetal tissues.

The serosal basement membrane, however, does not seem to demonstrate the same characteristics as other basement membranes. Although it becomes visible by day 14, it seems to degenerate by day 20, at which time its outline becomes hazy. It is PAS-positive, though less intensely so than the visceral basement membrane, and contains collagen, some reticular fibers and a small amount of elastic tissue which is not histochemically similar to elastic tissues located elsewhere (Wislocki and Padykula, 1953).

Function of the Visceral Yolk-Sac

Prior to 1927, when Brunschwig proposed the idea that the visceral yolk-sac is physiologically a placenta, it was customary to consider the chorio-allantoic placenta as the primary organ for bringing

nourishment to and taking waste from the developing embryo and growing fetus. Everett (1935) predicted that the proximal yolk-sac is at least as functionally important as the chorio-allantoic placenta. Twelve years later, Noer and Mossman (1947) suggested that due to its unique morphology, the proximal yolk-sac functions in a substantially different way than the chorio-allantoic placenta and is, therefore, complementary, rather than supplementary, to the chorio-allantoic placenta.

The possible functions of the visceral yolk-sac may be divided into at least two broad categories. First, it may protect the embryo from physical or chemical trauma and second, it may participate in the transfer of material between mother and embryo.

The Visceral Yolk-Sac as an Organ of Protection

Brambell *et al.* (1951) published data indicating that homologous gamma-globulin passed through the rabbit yolk-sac from the maternal side to the fetal side, but that heterologous gamma-globulins did not. This led to the question of whether or not the same phenomenon occurs in the rat. Ferm *et al.* (1959) were able to study the distribution of homologous and heterologous types of proteins in pregnant rats by combining them with a diazotized dye. Both protein-dye complexes were found concentrated in the vitelline epithelium at all tested stages of gestation, but neither homologous nor heterologous proteins were found within the embryo itself. There was, however, a distinct difference in the maternal distribution such that the heterologous protein-dye complex was not distributed as equally as the homologous protein-dye complex. This indicated that there were indeed two different proteins, but that the yolk-sac seemed to

treat them as one. Although these investigators speculated that the lack of color in the embryo and the apparent dense color in the yolk-sac indicated that the yolk-sac was protecting the embryo from the proteins, it is entirely possible that the yolk-sac contains the enzymatic machinery necessary to split the protein-dye complex. Under these circumstances, a false representation of the situation could easily have been achieved.

Two other studies have indicated that at least one of the functions of the visceral wall of the yolk-sac is protection. Ferm and Beaudoin (1960) demonstrated that yolk-sacs under _in vitro_ conditions accumulate and store both heterologous and homologous proteins in the same manner as _in vivo_ yolk-sacs. The conclusion was that the yolk-sac has an intrinsic blockade mechanism useful to the embryo as a protective device.

Finally, using a known teratogenic agent, Wilson _et al_. (1959) also concluded that the yolk-sac serves a protective function. Trypan blue, an azo dye, is teratogenic in the rat before the end of the eighth day of gestation. At the same time that the embryo becomes completely enveloped by the visceral yolk-sac, the teratogenicity of trypan blue is markedly reduced. When injected after day 8, the dye is absent from the embryo proper and is absorbed and stored by the vitelline epithelium. It was therefore suggested that the immobilization of trypan blue by the yolk-sac protects the embryo from a teratogenic stimulus. The efficiency of this proposed protective mechanism, however, is very low, for even when the dye is administered to pregnant rats at day 9, the

incidences of both embryonic malformation and resorption are significantly higher than the rate of spontaneous abnormality.

The Visceral Yolk-Sac as an Organ of Transfer

On the basis of pure morphology and biochemistry, the most likely and best documented function of the visceral yolk-sac is the transfer of materials between mother and embryo. Anatomical studies have indicated that the vitelline epithelium is composed of cells having characteristics similar to the absorbing cells of the intestinal villus and the active cells of the proximal convoluted tubule of the kidney, e.g., dense microvillus border, apical canaliculi, glycocalyx. In addition, the villous region of the visceral yolk-sac is remarkably similar to the mucosa of the small intestine, having an absorbing epithelium (the vitelline epithelium), a basement membrane (the visceral basement membrane) and a vascularized lamina propria (the mesenchymal layer containing vitelline blood vessels).

The closed vitelline circulatory pattern would also indicate the possible importance of an absorptive function. The vitelline blood vessels are conveyed between embryo and yolk-sac by the vitelline stalk which is analogous to the umbilical cord carrying blood vessels to and from the chorio-allantoic placenta. The vitelline artery is a branch of the embryonic aorta while the vitelline vein empties into the umbilical vein within the embryo. There is a dense capillary network in the villous region of the yolk-sac, a less dense network in the antimesometrial hemisphere and a totally avascular area at the hilus of the chorio-allantoic placenta.

The vitelline artery approaches the most mesometrial part of the villous region, penetrates the mesothelial layer and serosal basement membrane and divides into two main trunks, both of which are larger in diameter than their parent vessel. These trunks then divide into many branches which ramify throughout the mesenchymal layer. The probable physiological significance of the larger branches from the main arterial channel is to decrease the velocity of blood flow through the yolk-sac, thereby increasing the time available for absorption (or secretion) of materials (Bőe, 1951).

One of the earliest attempts to characterize placental function was in 1922, when Shimidzu injected 23 different dyes into pregnant rats and mice for the purpose of ascertaining placental permeability. Using the presence of color in fetal tissue as the criterion of permeability, it was concluded that the permeability of the dyes paralleled the colloidal state of their solution and their ability to diffuse through a gel. Thus, the placenta would act as an unselective ultrafilter, permitting the passage of small molecules while inhibiting the passage of larger ones. This was followed by other experiments which suggested that the proximal yolk-sac has the ability to absorb and transfer iron (Brunschwig, 1927; Everett, 1935) and fats (Everett, 1935; Koren and Shafrir, 1964).

In an attempt to correlate structure with function, Padykula and Wilson (1960) suggested that with an apparent ultrastructural degeneration of the yolk-sac at day 15, there was a steady decrease in the ability of the visceral endoderm to absorb radioactive vitamin B_{12}-intrinsic factor complex. Even though there was a fifty-fold increase

in yolk-sac weight between day 12 and term, there was only a five-fold increase in absorptive capacity. In addition, Jollie (1964) noted that the visceral endoderm was not labeled with tritiated thymidine between days 16 and 20, and that this might indicate a process of aging.

Alternatively, however, the previously noted sharp rise in the activity of certain enzymes at day 14 (Padykula, 1958) could indicate a greater functional capacity when the yolk-sac becomes exposed to the uterine lumen. Brambell and his colleagues have been able to show that the yolk-sac of the rabbit is capable of absorbing and transferring antibodies to the fetus at a late stage in gestation (Brambell et al., 1951; Brambell, 1958). Halliday (1955) continued the work using rats and found antibody absorption by the proximal yolk-sac at day 17. Finally, Brambell and Halliday (1956), by ligating the vitelline vessels, demonstrated that the vitelline epithelium and its underlying vascular system were partly responsible for antibodies penetrating the embryo.

In addition, Deren et al. (1966a) noted that the rabbit yolk-sac has the ability to concentrate vitamin B_{12} and further demonstrated that the rabbit yolk-sac develops an active transport system for certain amino acids at day 20 of the 32-day gestation period (Deren et al., 1966b). Lambson (1966) was able to infer the transfer of ferritin across the proximal yolk-sac of the rat from electron micrographs and Luse (1958), using other particulate matter, suggested that the rabbit yolk-sac absorbs those particles by pinocytosis.

It appears therefore that the visceral yolk-sac of the rat is a dynamic organ having the ability to pursue certain functions which are undoubtedly vital to normal embryonic development and fetal growth.

Statement of the Problem

From both morphological and biochemical evidence, it would seem that the yolk-sac participates in an absorptive function such that nutrients or other molecules enter the vitelline epithelium and pass through the epithelial cytoplasm, the visceral basement membrane, the basement membrane of the endothelium lining the vitelline capillaries, the endothelium itself and, finally, enter the vitelline circulation to be circulated throughout the embryo. It is unlikely, though as yet unproven, that material would penetrate the total width of the yolk-sac and pass to the embryo by simple diffusion across the exocelom, amnion and amniotic fluid (Wislocki, 1921).

Proceeding on the hypothesis that the yolk-sac does indeed have an absorptive function, it became of interest to test first, the ability of the yolk-sac to absorb various radioactively-labeled ions and second, the effect, if any, of a potent teratogenic agent on the concentrating ability of the yolk-sac.

Trypan blue was chosen as the teratogenic agent because (1) it is accumulated in the vitelline epithelium, but not in the chorionic villi (Everett, 1935), and it has never been found to penetrate into embryonic rat tissue *per se*; (2) its administration to pregnant rats results in a high incidence of severely congenitally malformed fetuses and (3) it may be used as a possible model system for analyzing the complex interrelationships between the genome of the developing embryo and its microenvironment.

Goldmann (1909) was one of the first to note that when a pregnant rat was vitally stained with trypan blue, the dye was apparently

concentrated in the proximal endoderm of the yolk-sac (Everett, 1935). He also suggested that with the initiation of pregnancy, the dye is released by the maternal reticulo-endothelial system and liver. Following this release it is free to circulate in the blood vascular system (Wislocki, 1921). Zaretsky (1910), the first to use trypan blue in avian embryos, also noted that the yolk-sac of a developing chicken has the ability to absorb the dye and, furthermore, has the ability to prevent the dye from penetrating the embryonic tissue proper (Hanan, 1927).

Apparently unaware that trypan blue concentrates in the yolk-sac of the rat, Gillman et al. (1948) injected the dye into pregnant rats under the hypothesis that particulate matter or abnormal proteins in the maternal circulatory system (since trypan blue is adsorbed to serum albumin, Rawson, 1943) could play a role in the production of congenital malformations. Indeed, their results indicated that the dye was teratogenic when administered during pregnancy and that the central nervous system was usually the most severely affected system of the embryo.

Other investigators who have pursued the matter further have confirmed the teratogenicity of trypan blue in the rat (Hogan, et al., 1950), mouse (Hamburgh, 1952), rabbit (Harm, 1954) and chicken (Beaudoin and Wilson, 1958). Wilson (1955) noted that the most susceptible period for trypan blue-induced teratogenesis in the rat is on days 7, 8 and 9, the period during which the central nervous system is undergoing its critical period of differentiation and development.

By correlating the distribution of trypan blue and the known effect of the dye in producing abnormal young, Lloyd and Beck (1966)

and Beck *et al*. (1967) have suggested that trypan blue acts by inhibiting the passage of vital substances across the yolk-sac. The supposition that the yolk-sac is the site of action of trypan blue is a reasonable one based on the generality that a teratogen will act on only one or a combination of three possible locations: the embryo, the mother, or the organ intervening between the two. The embryo seems to have been eliminated as a possible site of action since no dye apparently penetrates into its substance. The mother seems also to have been eliminated since trypan blue is highly teratogenic when injected into the yolk of a developing chicken embryo.

The present experiments were therefore initiated to determine if trypan blue under both *in vitro* and *in vivo* circumstances does indeed have an effect on the absorptive ability of the yolk-sac. To this end, three pertinent questions were asked:

1. Are the yolk-sacs from normally developing rat embryos capable of absorbing ions?

2. Is there a difference between the amount of material that can be taken up by normal control and trypan blue-treated yolk-sacs?

3. Does a nonteratogenic azo dye, Niagara blue 2B (Beaudoin and Pickering, 1960), which also localizes in the visceral endoderm, but is excreted more rapidly (Lloyd and Beck, 1966), have an effect on ion uptake?

MATERIALS AND METHODS

Care and Breeding of Animals

Virgin, black-hooded female rats of the Long-Evans strain, weighing between 60 and 100 g, were obtained from Research Animals, Inc.[2] The animals were housed in wire-bottomed cages in a windowless, well-ventilated room with an alternating 12-hour light-dark cycle. All animals were fed a diet consisting of stock laboratory chow[3] and tap water *ad libitum*. The ration was supplemented twice weekly with lettuce and once weekly with canned horse meat.

Late every afternoon, a smear of the vaginal contents of each female weighing between 180 and 240 g and 80 to 120 days of age was examined by light microscopy to detect those animals in proestrus (Long and Evans, 1922). Each proestrus female was caged overnight with a mature male of the same strain. The presence of sperm in a vaginal smear at 10:00 AM the following morning was considered as day 0 of pregnancy.

Incidence of Gross Malformation at Term

Twenty-two pregnant rats were given a single, subcutaneous injection of 1.8 per cent aqueous trypan blue[4] at a dosage of 1 mg/6 g

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[2]Pittsburgh, Pennsylvania.

[3]Purina Rat Chow, The Ralston-Purina Co., St. Louis, Missouri.

[4]Specially purified and donated through the courtesy of Mr. Floyd Greene of the Matheson, Coleman and Bell Division of the Matheson Co., Inc., Norwood, Ohio.

maternal body weight (167 mg/kg) at 10:00 AM on day 8 of gestation. Another group of 12 pregnant rats was similarly injected with Niagara blue 2B[5,6] and a third group of 12 females was untreated.

At day 20 of gestation (1 day before parturition) all females were anesthetized with ether and killed by cervical dislocation. The intact uterus was removed, opened along the antimesometrial border and the fetuses were dissected free of their associated membranes. All living fetuses were examined for gross external malformations and placed in Bouin's fluid[7] for later freehand sectioning (Wilson, 1965) and the identification of any gross internal malformations.

Preparation of the Culture Medium for Ion Uptake in Vitro

The culture medium was designed for use in manometric studies of oxygen consumption by normally and abnormally developing rat embryos (Netzloff et al., 1968). It consisted of bovine serum,[8] chicken embryo extract ultrafiltrate[9] and a phosphate-Ringer buffer[10] modified after Kosan and Burton (1966) in a ratio of 3:1:1.

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[5]Also named Benzo Blue 2B.

[6]Obtained from the Hartman-Leddon Co., Philadelphia, Pennsylvania.

[7]See Appendix A.

[8]Obtained from Microbiological Associates, Inc., Bethesda, Maryland.

[9]Obtained from Microbiological Associates, Inc., Bethesda, Maryland. Chicken embryo homogenized in an equal volume of Gey's balanced salt solution with 100 units each of penicillin and streptomycin added per ml before ultrafiltration.

[10]See Appendix B.

The serum and ultrafiltrate were both purchased as single lot numbers in 100 ml bottles for the former and 20 ml bottles for the latter and stored at -55° C prior to use. The buffer was mixed in advance and stored at 5° C. On the day of an experiment, the culture medium was freshly prepared and D-glucose[11] was added to a final concentration of 1 mg/ml. After the culture medium was warmed to a temperature of 39° C, it was aerated for 2 minutes with air passed through a water trap.

Twenty ul of a previously prepared stock solution of $^{45}Ca^{++}$,[12] $^{35}SO_4^{--}$[13] or $^{22}Na^{+}$[14] were added to each milliliter of an aliquot of the aerated culture medium. The final activities of ions were 0.210 uC/ml for calcium, 0.072, uC/ml for sulfate and 0.019 uC/ml for sodium. Enough labeled medium to completely submerse the tissue preparation was placed into plastic, disposable 30 ml beakers and warmed to 38° - 40° C. A second aliquot of the aerated culture medium was stored at 39° C in small petri dishes.

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[11]Obtained from the Fisher Scientific Co., Fair Lawn, New Jersey.

[12]Obtained from Nuclear-Chicago Corp., Des Plaines, Illinois. Calcium-45 as calcium chloride in aqueous solution with a specific activity of 8.73 uC/ug.

[13]Obtained from Nuclear-Chicago Corp., Des Plaines, Illinois. Sulfur-35 as carrier-free sulfate in aqueous solution.

[14]Obtained from Nuclear-Chicago Corp., Des Plaines, Illinois. Sodium-22 as sodium chloride in aqueous solution with a specific activity of 9 uC/ug.

Preparation of Tissue for in Vitro Uptake of Radioisotopes

Thirty-four normal control, 34 trypan blue and 19 Niagara blue 2B injected pregnant females were stunned by a blow to the head at 10:00 AM of days 12, 13 and 14 of gestation. The animals were killed by cervical dislocation, the uterus removed and placed in a petri dish containing warmed, but unlabeled, medium. The number of implantation sites and resorbing sites were noted and recorded.

One implantation site was separated from the remaining intact uterus and transferred to another petri dish which contained warmed, unlabeled medium. After opening the implantation site along the anti-mesometrial wall, the decidua capsularis and parietal yolk-sac were dissected free of the chorio-allantoic placenta and visceral yolk-sac. A silk ligature was tied around the umbilical vessels at the point where they enter the chorio-allantoic placenta (Fig. 3). The placenta and uterus were then separated from the ligated proximal yolk-sac which remained as a complete and vascularized membrane surrounding the embryo (Fig. 4).

The proximal yolk-sac, vitelline vessels and embryo were examined under a dissecting microscope to be certain that (1) there was no puncture wound in the proximal yolk-sac, (2) no vitelline vessels were ruptured and (3) the embryo had a beating heart which perfused the vitelline vessels with blood. The latter parameter was used as the criterion for diagnosing embryonic viability in all stages of the experiment. No more than 6 embryos from each pregnant female were utilized.

Fig. 3.—Embryo-in-yolk-sac preparations prior to the removal of the chorio-allantoic placenta and uterine muscle.

A. Day 12 preparation. 8X.

B. Day 13 preparation. 8X.

1. Vitelline vessels
2. Visceral yolk-sac
3. Embryo
4. Silk ligature
5. Chorio-allantoic placenta
6. Reflected uterine muscle

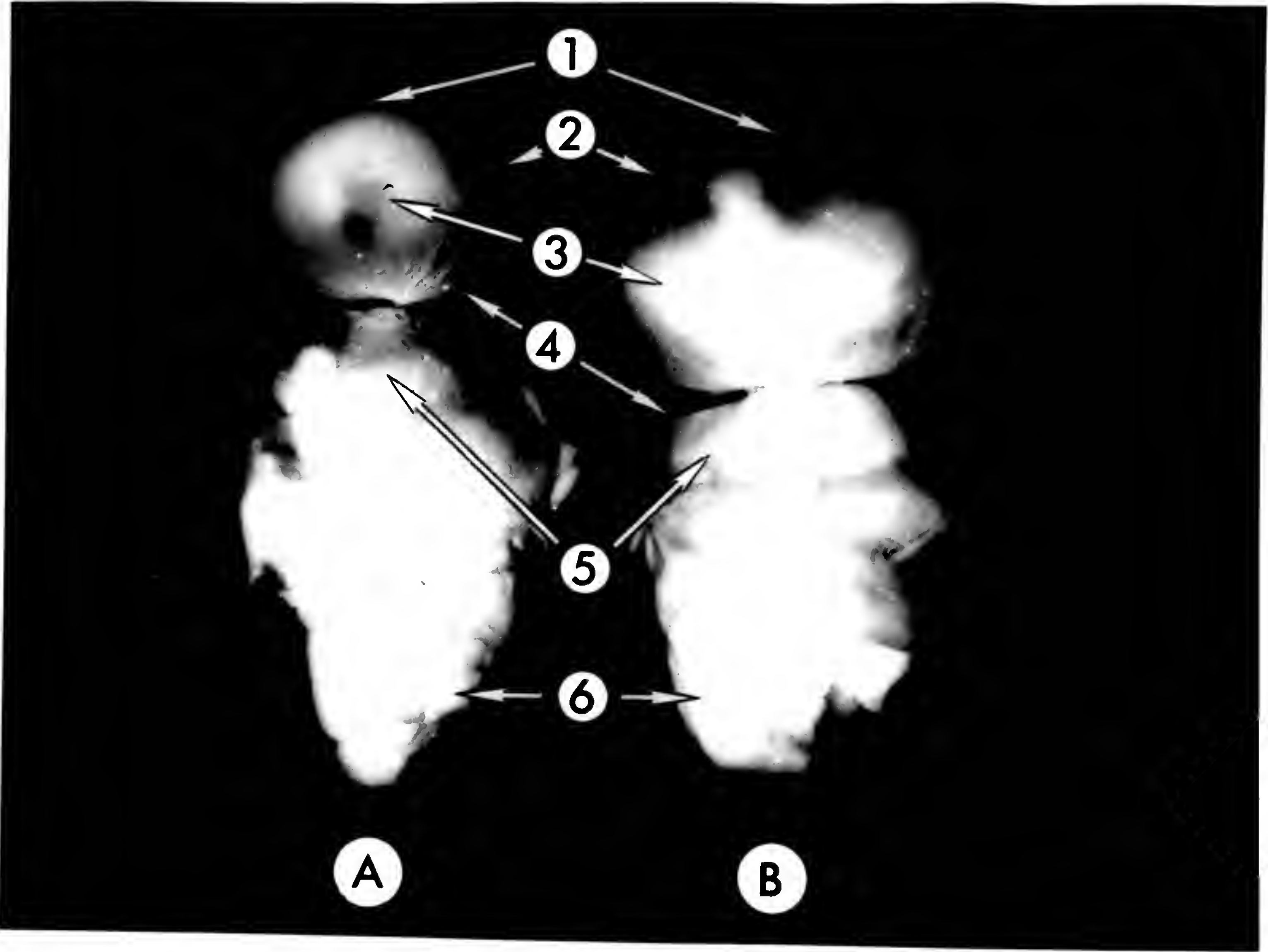
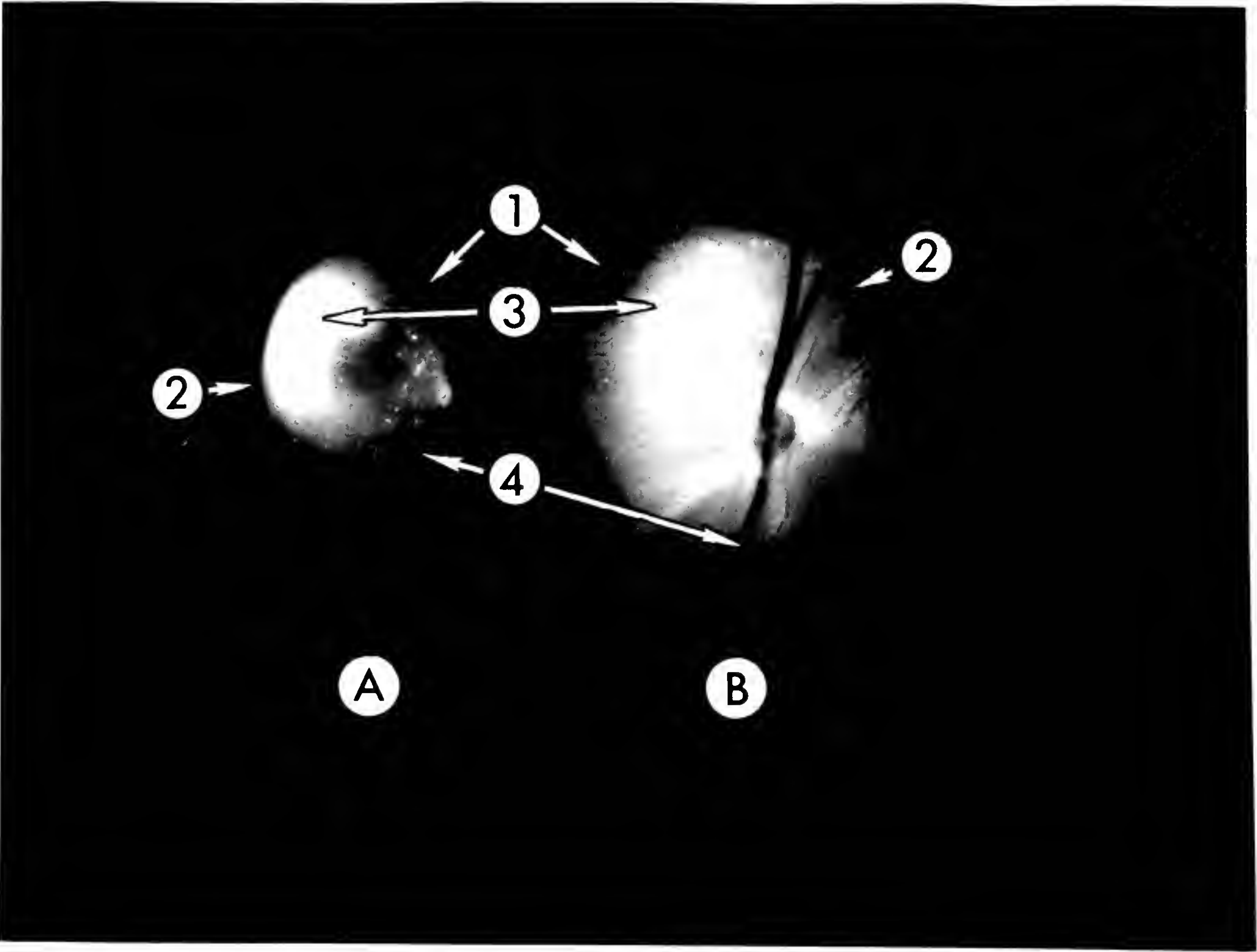

1
2
3
4
5
6
A
B

Fig. 4.—Embryo-in-yolk-sac preparations after the removal of the chorio-allantoic placenta and uterine muscle.

A. Day 12 preparation. 8X.

B. Day 13 preparation. 8X.

1. Visceral yolk-sac
2. Vitelline vessels
3. Embryo
4. Silk ligature


1
2
3
2
4
A
B

Exposure of Tissues to Tagged Ions in Vitro

The embryo-in-yolk-sac preparation was transferred to incubation medium containing one of the radioactively labeled ions. All manipulations of the preparations were done by using the free ends of the ligature and care was taken to avoid brushing the preparation against the walls of any of the vessels.

After 60 minutes of incubation, the preparations were removed from the tracer-bearing medium, examined for the presence of a heart beat and rinsed several times in 0.9 per cent saline. The proximal yolk-sacs were separated from the embryos and each was rinsed 5 times in saline. The tissues were transferred to individual 10 x 75 mm test tubes containing 0.25 ml of concentrated nitric acid and dissolved over a low flame. The resulting solution was placed on a tared, stainless steel, ringed planchet, dried for 24 hours at 70° C and for another 24 hours at 130° C. The planchets were weighed and then counted for radioactivity with a well-shielded, halogen-quenched Geiger-Müller tube. Corrections were made for the decay rates of the isotopes and the results were expressed as counts per minute per preparation (cpm/prep) and counts per minute per mg dry weight (cpm/mg).

Ion Uptake in Vivo

To determine whether any differences in the uptake of radioactively labeled ions between normally and abnormally developing embryos could be detected under *in vivo* conditions, autoradiographic procedures were employed.

For this purpose, 8 pregnant rats were divided into 2 groups of 4 each. One group was left untreated, while each rat in the other

group was given a single, subcutaneous injection of 1 mg trypan blue/ 6 g maternal body weight on day 8 of gestation. At 10:00 AM of day 13, each animal was injected intraperitoneally with 10 uC of carrier-free $^{35}SO_4^{--}$/g body weight (Kochhar and Johnson, 1965). Two animals, one from each group, were killed by cervical dislocation at 15 minutes, 30 minutes, 1 hour and 3 hours. The implantation sites were removed and fixed in alcohol-formalin, dehydrated through increasing concentrations of ethyl alcohol, cleared in terpineol and embedded in paraffin. The tissue block was then serially sectioned at 5 u and placed on pre-treated[15] 1" x 3" glass slides.

Autoradiography was done by a dipping method (Messier and Leblond, 1957) in a completely light-proof darkroom under a No. 2 Wratten safelight. Eastman Kodak NTB 3 emulsion gel was placed in a dipping container and warmed to a temperature of 40° - 45° C in a water bath. After the emulsion had liquefied (1 hour), the slides, held by the label end, were individually dipped, once each, for 1 - 2 seconds. The slides were removed from the emulsion and their backs wiped dry. They were then placed on a slide rack in a horizontal position and allowed to dry at room temperature for 1 hour. The slides were inserted into black, plastic slide boxes containing granular calcium chloride or silica gel as drying agents and separated from one another by plain

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[15]The glass slides were treated to provide for better adhesion between the photographic emulsion and tissue sections. After being soaked in dichromate solution for several hours, they were rinsed in tap water, dipped in acetone and air dried. They were then immersed in a warm solution of 0.5 per cent gelatin and 0.05 per cent chrom-alum in distilled water and dried at room temperature in a covered staining dish (Boyd, 1955).

glass slides. The boxes were sealed with black tape and placed in a vertical position in a dry atmosphere so that the emulsion sides faced down.

After 4 days of exposure, the slides were developed in the darkroom in the following manner:

Kodak developer (type D 19)	5 min
Kodak stop bath (type SB 5a)	15 sec
Kodak acid fixer	10 min
Water rinse	15 min

They were immediately stained with hematoxylin (4 min) and eosin (12 sec), dehydrated through a graded series of ethyl alcohols, cleared in xylene and permanently mounted in HSR[16] mounting medium. The sections were examined by light microscopy and comparisons of the number of developed granules were made.

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[16]Obtained from the Hartman-Leddon Co., Philadelphia, Pennsylvania.

RESULTS

Teratogenic Action of Trypan Blue and of Niagara Blue 2B

The administration of a single, subcutaneous injection of trypan blue at a dosage of 167 mg/kg maternal body weight on the eighth day of pregnancy in the rat results in a high incidence of congenitally malformed fetuses (Table 1). The same treatment with Niagara blue 2B induces a much lower rate of malformation which is nonetheless, significantly greater than the spontaneous incidence of malformation seen in normal control animals. From 12 control animals sacrified 1 day before parturition, 99 per cent of the fetuses were living and structurally normal, while 1 per cent had been resorbed. The presence of trypan blue resulted in a resorption rate of 46 per cent and a malformation rate of 62 per cent among the living fetuses. In many cases, the young bore multiple congenital abnormalities of varying severity. The most common site of malformation was the central nervous system and included defects such as anencephaly, exencephaly, meningocele, meningomyelocele, anophthalmia, microphthalmia and occasionally, hydrocephaly and spina bifida. Micrognathia, microstomia, cleft palate, situs inversus and kinky tail were only infrequently encountered.

The injection of Niagara blue 2B resulted in a 7 per cent resorption rate and a gross malformation rate of 2 per cent in the living young. These defects were also of the central nervous system and consisted of anophthalmia and hydrocephaly.

TABLE 1

INCIDENCE OF MALFORMATION[a]

Treatment	Dose (mg/kg)	Number of Females	Number of Sites	Number Living (Per cent)	Number Resorbing (Per cent)	Survivors: Number Normal (Per cent)	Survivors: Number Abnormal (Per cent)
Control[b]	-	12	136	134 (99)	2 (1)	134 (100)	0
Trypan Blue[c]	167	22	240	130 (54)	110 (46)	49 (38)	81 (62)
Niagara Blue 2B[d]	167	12	144	134 (93)	10 (7)	131 (98)	3 (2)

[a]Animals were sacrificed on day 20 of gestation.

[b]Maternal animals left untreated.

[c]A single subcutaneous injection of a 1.8 per cent aqueous solution of the dye administered on day 8 of gestation.

[d]*Ibid.*

Effects of Azo Dyes on the in Vitro Uptake of Labeled Ions

Dry Weights of Yolk-Sacs and Embryos

For the purpose of ascertaining the effects of trypan blue and Niagara blue 2B on the general growth of yolk-sacs and embryos, the dry weights of the tissues were compared (Table 2, Figs. 5 and 6). Table 2 shows the mean dry weights and standard errors in milligrans for control and experimental yolk-sacs and embryos at days 12, 13 and 14 of gestation. On a semi-logarithmic graph (Fig. 5) of the dry weights, the curves approximate the straight lines indicative of growth curves in general. At days 12 and 13, yolk-sacs of the control preparations weigh significantly more than the corresponding trypan blue-treated group, while they do not statistically differ from those treated with Niagara blue 2B. By day 14, there is an apparent recovery of yolk-sac weights as there are no differences among the control and experimental values. However, in the case of embryonic dry weights, day 13 control embryos weighed more than the trypan blue-treated, while an increase in embryonic weight at day 14 was noted in the Niagara blue 2B group.

Figure 6, which expresses experimental yolk-sac and embryonic dry weights in terms of percentages of control values demonstrates the apparent recovery of yolk-sac weight by day 14. Although both groups of treated yolk-sacs approach control values, the trypan blue-treated group demonstrates a greater rate of recovery. No such recovery phenomenon can be described for embryonic dry weights.

TABLE 2

DRY WEIGHT OF YOLK-SACS AND EMBRYOS[a]

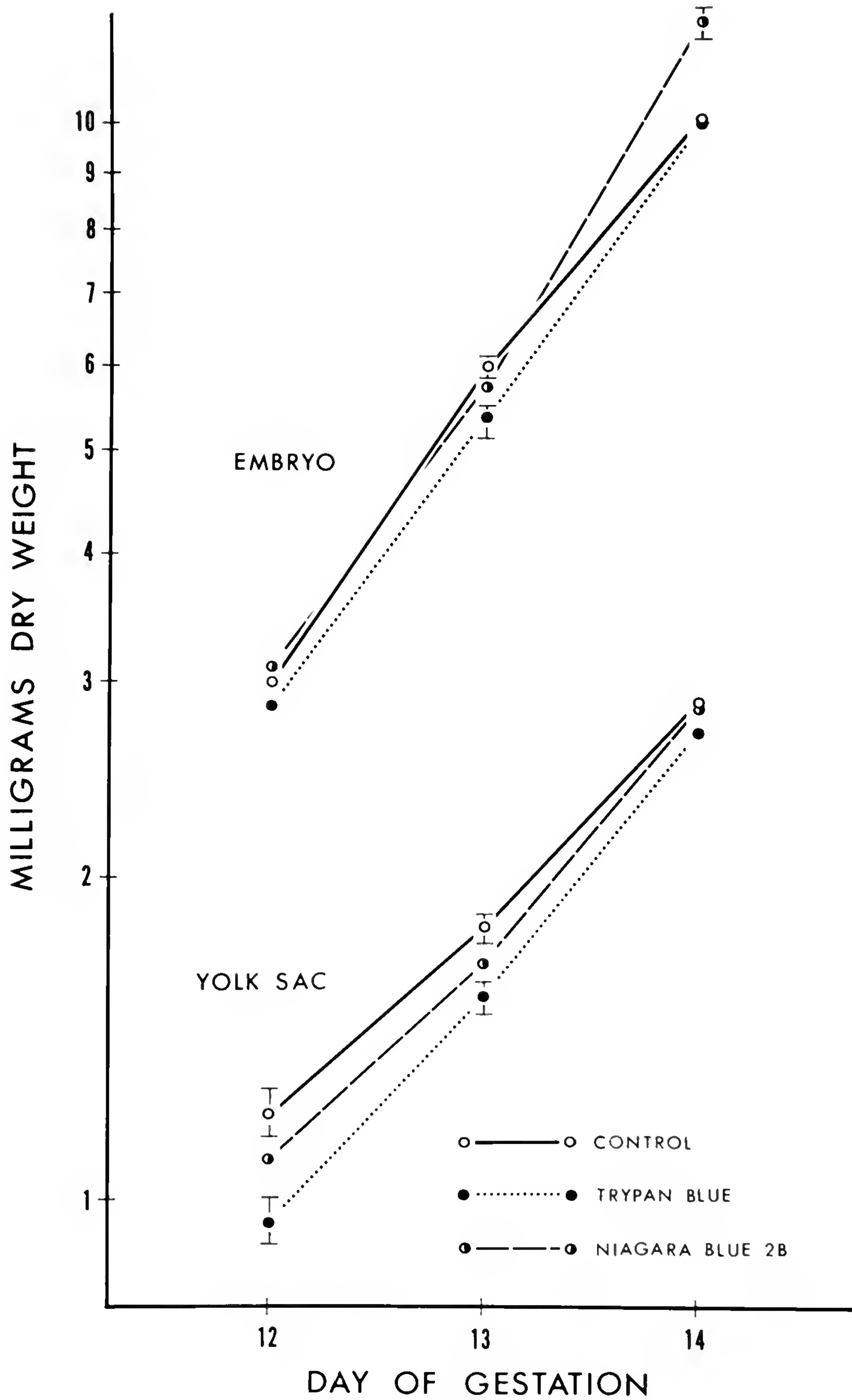
Treatment[b]	Day 12	Day 13	Day 14
Yolk-Sac			
Control	1.21 ± 0.08 (62/13)[c]	1.80 ± 0.06 (67/12)	2.91 ± 0.09 (61/11)
Trypan Blue	0.94 ± 0.05 (59/12)	1.55 ± 0.05 (54/11)	2.73 ± 0.08 (41/11)
Niagara Blue 2B	1.09 ± 0.09 (40/7)	1.66 ± 0.06 (35/6)	2.92 ± 0.08 (34/6)
Embryo			
Control	3.04 ± 0.09 (61/13)	5.96 ± 0.17 (65/12)	10.99 ± 0.38 (61/11)
Trypan Blue	2.89 ± 0.10 (58/12)	5.32 ± 0.18 (52/11)	10.92 ± 0.38 (40/11)
Niagara Blue 2B	3.14 ± 0.10 (40/7)	5.73 ± 0.13 (36/6)	12.50 ± 0.31 (34/6)

[a]Dry weight in milligrams, mean ± standard error.

[b]See legend in Table 1 for details of treatment.

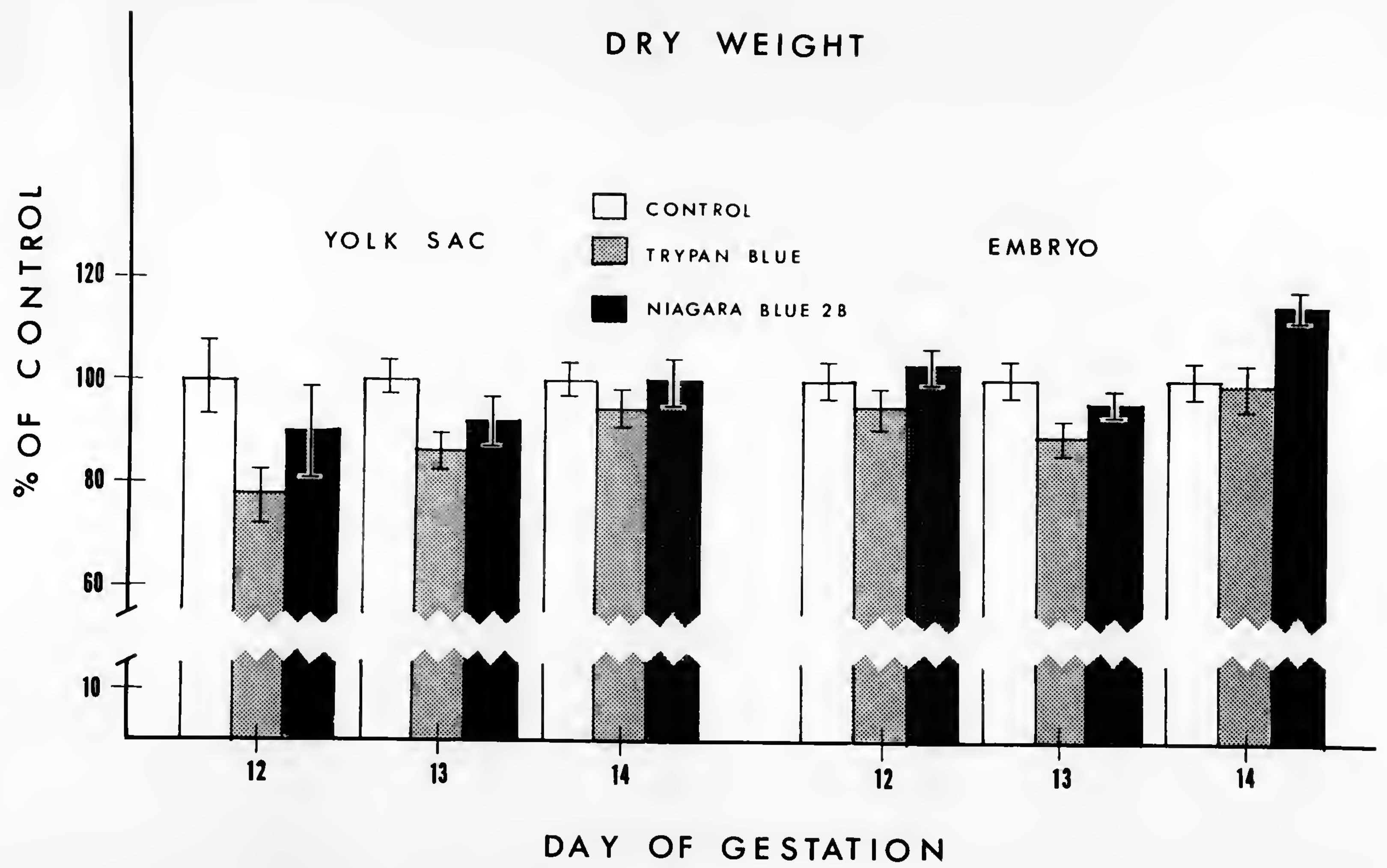
[c](Number of preparations/number of mothers).

Fig. 5.—Semi-logarithmic graph comparing mean tissue dry weights with gestational age. Vertical bars represent standard errors and are only present when a significant difference ($p < 0.05$) exists. See Table 2 for numerical data.



MILLIGRAMS DRY WEIGHT
10
9
8
7
6
5
4
3
2
1
EMBRYO
YOLK SAC
CONTROL
TRYPAN BLUE
NIAGARA BLUE 2B
12
13
14
DAY OF GESTATION

Fig. 6.—Bar graph depicting dye-treated tissue dry weights as per cent of control. Control values set at 100 per cent. Vertical bars represent standard errors. See Table 2 for numerical data.


DRY WEIGHT
YOLK SAC
EMBRYO
CONTROL
TRYPAN BLUE
NIAGARA BLUE 2B
% OF CONTROL
120
100
80
60
10
12
13
14
12
13
14
DAY OF GESTATION

The Uptake of $^{45}Ca^{++}$

Table 3 indicates the uptake of $^{45}Ca^{++}$ by control and trypan blue-treated yolk-sacs and embryos on days 12, 13 and 14 of gestation. In a graphic comparison between control and treated tissue (Fig. 7), no significant differences in ion uptake on a preparation basis can be detected. However, the manner in which these curves approach a straight line and their slopes would tend to indicate that the rate at which the label is taken up by the individual preparation is a function of its dry weight.

A more realistic and possibly more accurate representation of the amount of $^{45}Ca^{++}$ absorbed is shown in Figure 8. The logarithm of the specific activity of $^{45}Ca^{++}$ (cpm/mg dry weight) is plotted against the day of gestation. At day 13, control yolk-sacs demonstrate a decreased capacity to absorb $^{45}Ca^{++}$ when compared to day 12, while the specific activity is shown to undergo a rapid increase by day 14. Trypan blue-treated yolk-sacs also show the same variation in specific activity with gestational age, but the changes from day to day are considerably less than control. More interesting, however, is that the experimental yolk-sacs have a significantly greater specific activity than the corresponding controls at day 13, while by day 14, the controls are able to absorb more $^{45}Ca^{++}$ than the treated group.

No such pronounced changes in the specific activities of the control and experimental embryos were observed. Although the day 13 and 14 experimental embryos bear the same relationship to the controls as in the cases of the corresponding yolk-sacs, no statistically significant differences were noted. Furthermore, a comparison between the

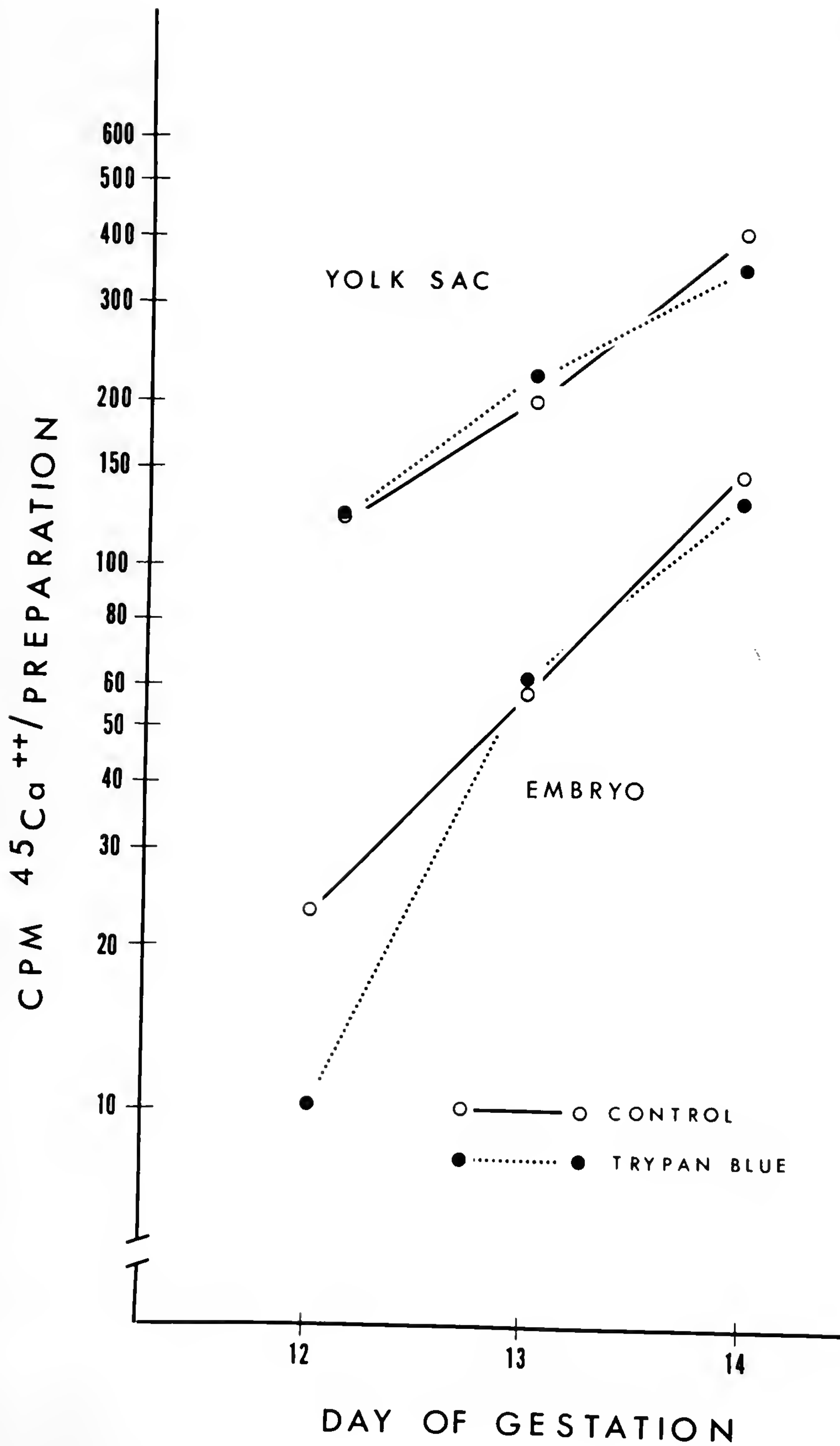
TABLE 3

ABSORPTION OF $^{45}Ca^{++}$ [a]

	CPM $^{45}Ca^{++}$/Preparation		CPM $^{45}Ca^{++}$/mg Dry Weight	
	Control	Trypan Blue	Control	Trypan Blue
Day 12				
Yolk-Sac	121 ± 6 (16/3)	120 ± 9 (14/3)	136 ± 6 (16/3)	144 ± 9 (14/3)
Embryo	23 ± 4 (16/3)	10 ± 6 (13/3)	7 ± 1 (16/3)	3 ± 2 (13/3)
Day 13				
Yolk-Sac	200 ± 10 (18/3)	223 ± 12 (15/3)	87 ± 4 (18/3)	126 ± 5 (15/3)
Embryo	59 ± 4 (16/3)	62 ± 11 (14/3)	9 ± 1 (16/3)	11 ± 2 (14/3)
Day 14				
Yolk-Sac	410 ± 43 (20/4)	352 ± 39 (10/3)	174 ± 12 (20/4)	132 ± 13 (10/3)
Embryo	150 ± 29 (19/4)	132 ± 34 (10/3)	16 ± 3 (19/4)	14 ± 3 (10/3)

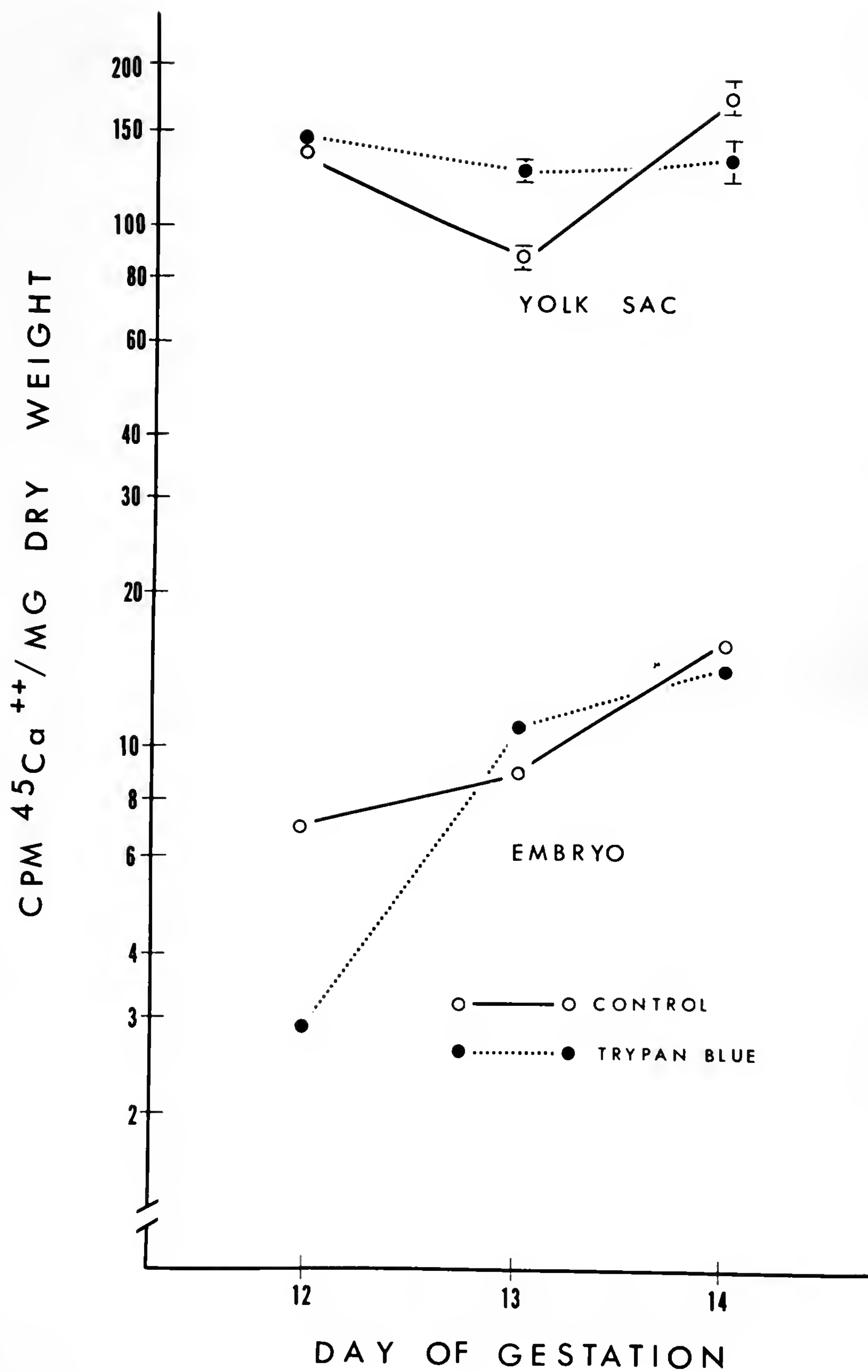
[a]See text and Table 1 for details of treatment. Data are expressed as mean ± standard error. Figures in parentheses indicate: (number of preparations/number of mothers).

Fig. 7.—Semi-logarithmic graph comparing mean tissue absorption of $^{45}Ca^{++}$ with gestational age. See Table 3 for numerical data.



600
500
400
300
200
150
100
80
60
50
40
30
20
10
CPM 45Ca ++/PREPARATION
YOLK SAC
EMBRYO
CONTROL
TRYPAN BLUE
12
13
14
DAY OF GESTATION

Fig. 8.—Semi-logarithmic graph comparing mean tissue $^{45}Ca^{++}$ specific activity with gestational age. Vertical bars represent standard errors and are only present when a significant difference ($p < 0.05$) exists. See Table 3 for numerical data.


200
150
100
80
60
40
30
20
10
8
6
4
3
2
CPM 45Ca ++/MG DRY WEIGHT
YOLK SAC
EMBRYO
CONTROL
TRYPAN BLUE
12
13
14
DAY OF GESTATION

uptakes of yolk-sacs and embryos in terms of both the preparation and the dry weight would indicate that a very small proportion of the $^{45}Ca^{++}$ that is available penetrates into the embryonic tissue proper.

The Uptake of $^{35}SO_4^{--}$

The means and their standard errors for the absorption of $^{35}SO_4^{--}$ by control and dye-treated tissues are shown in Table 4. Both yolk-sacs and embryos from the control and the two experimental groups demonstrate a progressive increase in labeling with gestational age and, therefore, with increasing tissue weight (Fig. 9). The slopes of the lines indicate that the rate of increase by the yolk-sacs between days 13 and 14 is less than the rate between days 12 and 13. Only those embryos from Niagara blue 2B-treated females show the corresponding change in slope. Furthermore, the actual amounts, i.e., cpm, of labeled $^{35}SO_4^{--}$ measured in yolk-sac and embryonic tissues are nearly equivalent.

A statistical comparison (Student's t-test) between the control and experimental groups at each day of gestation indicates that the day 12 Niagara blue 2B-treated yolk-sacs absorb a significantly ($p < 0.05$) greater amount of $^{35}SO_4^{--}$ than the controls. Compared with the control, trypan blue treatment results in a significantly greater amount of the tagged ion being absorbed by the yolk-sacs at day 13. Yolk-sacs laden with Niagara blue 2B show no difference in their ability to accumulate $^{35}SO_4^{--}$ when compared with control or with trypan blue treatment, while day 14 yolk-sacs absorb similar amounts of label regardless of treatment.

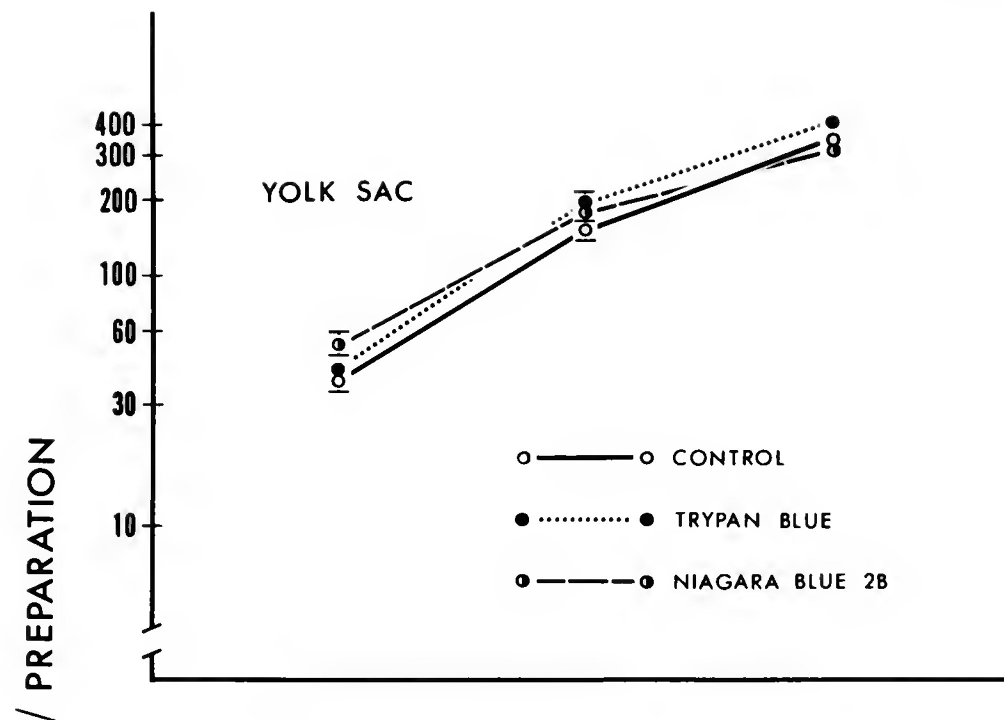
TABLE 4

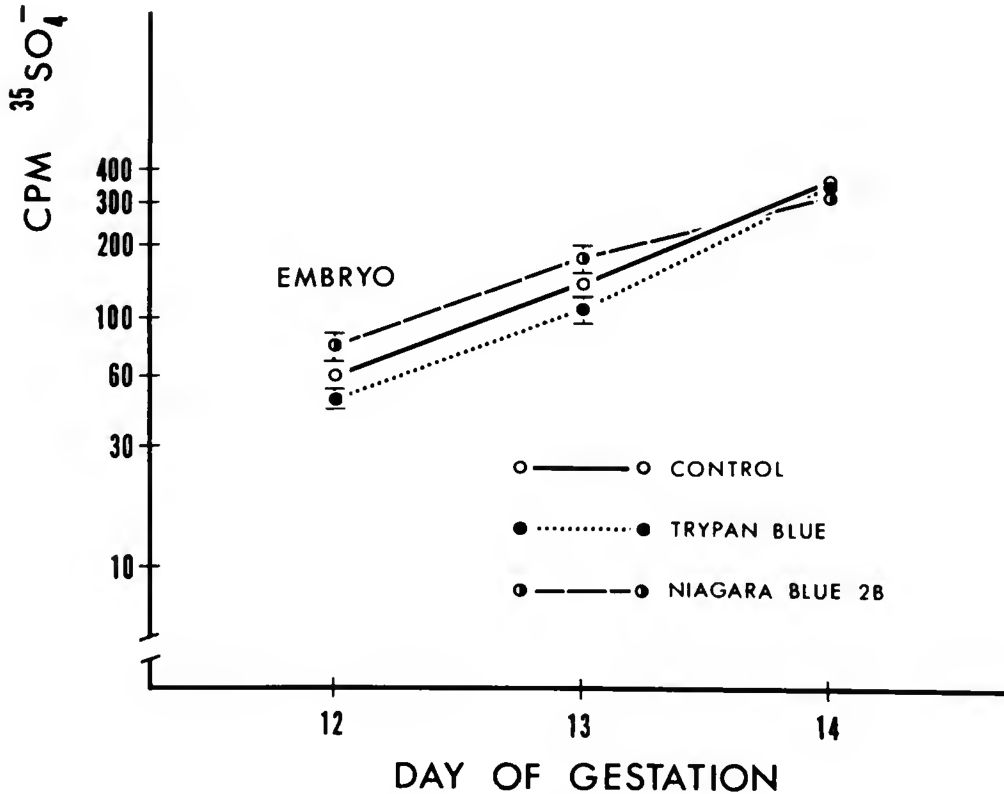
ABSORPTION OF $^{35}SO_4^{--}$ [a]

	CPM $^{35}SO_4^{--}$/Preparation			CPM $^{35}SO_4^{--}$/mg Dry Weight		
	Control	Trypan Blue	Niagara Blue 2B	Control	Trypan Blue	Niagara Blue 2B
Day 12						
Yolk-Sac	38 ± 3 (24/4)	43 ± 5 (19/4)	53 ± 4 (17/3)	21 ± 2 (24/4)	33 ± 4 (19/4)	34 ± 3 (17/3)
Embryo	60 ± 6 (24/4)	48 ± 3 (19/4)	77 ± 10 (17/3)	22 ± 3 (24/4)	18 ± 2 (19/4)	24 ± 4 (17/3)
Day 13						
Yolk-Sac	152 ± 11 (25/5)	194 ± 12 (20/4)	187 ± 22 (17/3)	112 ± 6 (25/5)	163 ± 18 (20/4)	118 ± 13 (17/3)
Embryo	137 ± 12 (25/5)	110 ± 13 (20/4)	176 ± 25 (18/3)	29 ± 3 (25/5)	24 ± 3 (20/4)	33 ± 5 (18/3)
Day 14						
Yolk-Sac	354 ± 29 (20/4)	411 ± 28 (13/4)	347 ± 56 (18/3)	116 ± 9 (20/4)	166 ± 13 (13/4)	118 ± 16 (18/3)
Embryo	356 ± 51 (21/4)	352 ± 61 (12/4)	326 ± 49 (18/3)	35 ± 5 (21/4)	36 ± 6 (12/4)	27 ± 4 (18/3)

[a] See text and Table 1 for details of treatment. Data expressed as mean ± standard error. Figures in parentheses indicate: (number of preparations/number of mothers).

Fig. 9.—Semi-logarithmic graph comparing mean tissue absorption of $^{35}SO_4^{--}$ with gestational age. Vertical bars represent standard errors and are only present when a significant difference ($p < 0.05$) exists. See Table 4 for numerical data.


CPM $^{35}SO_4^{--}$ / PREPARATION
400
300
200
100
60
30
10
YOLK SAC
CONTROL
TRYPAN BLUE
NIAGARA BLUE 2B


400
300
200
100
60
30
10
EMBRYO
CONTROL
TRYPAN BLUE
NIAGARA BLUE 2B
12
13
14
DAY OF GESTATION

Embryonic tissue also demonstrates no differences in its ability to accumulate the label at day 14. However, at both days 12 and 13, Niagara blue 2B treatment results in a significantly greater uptake than trypan blue treatment. The control values lie in between the experimental values and are not significantly different from them.

By comparing the specific activities of $^{35}SO_4^{--}$ (Fig. 10), it can be seen that the trypan blue-treated yolk-sacs incorporate significantly greater amounts of label than the corresponding controls. Yolk-sacs laden with Niagara blue 2B accumulate more of the ion at day 12 only. By day 13, they apparently begin to recover and this recovery is maintained to day 14. In all cases, yolk-sacs on day 12 exhibit a relatively low specific activity of $^{35}SO_4^{--}$ which increases greatly on day 13 and levels off by day 14.

No significant differences in specific activities between control and experimental embryos at any stage of gestation were noted. With the exception of the decrease in specific activity by Niagara blue 2B-treated embryos between days 13 and 14, all systems increased their uptake with gestational age.

The Uptake of $^{22}Na^+$

Table 5 summarizes the actual amounts and specific activities of $^{22}Na^+$ absorbed by control and experimental yolk-sacs and embryos on days 12, 13 and 14 of gestation. Figure 11 is a semi-logarithmic graph showing the amount of $^{22}Na^+$ absorbed per preparation as a function of gestational age. The yolk-sacs of all groups appear to be capable of absorbing more tagged ion with increased age and weight. At day 12, the

Fig. 10.—Semi-logarithmic graph comparing mean tissue $^{35}SO_4^{--}$ specific activity with gestational age. Vertical bars represent standard errors and are only present when a significant difference ($p < 0.05$) exists. See Table 4 for numerical data.

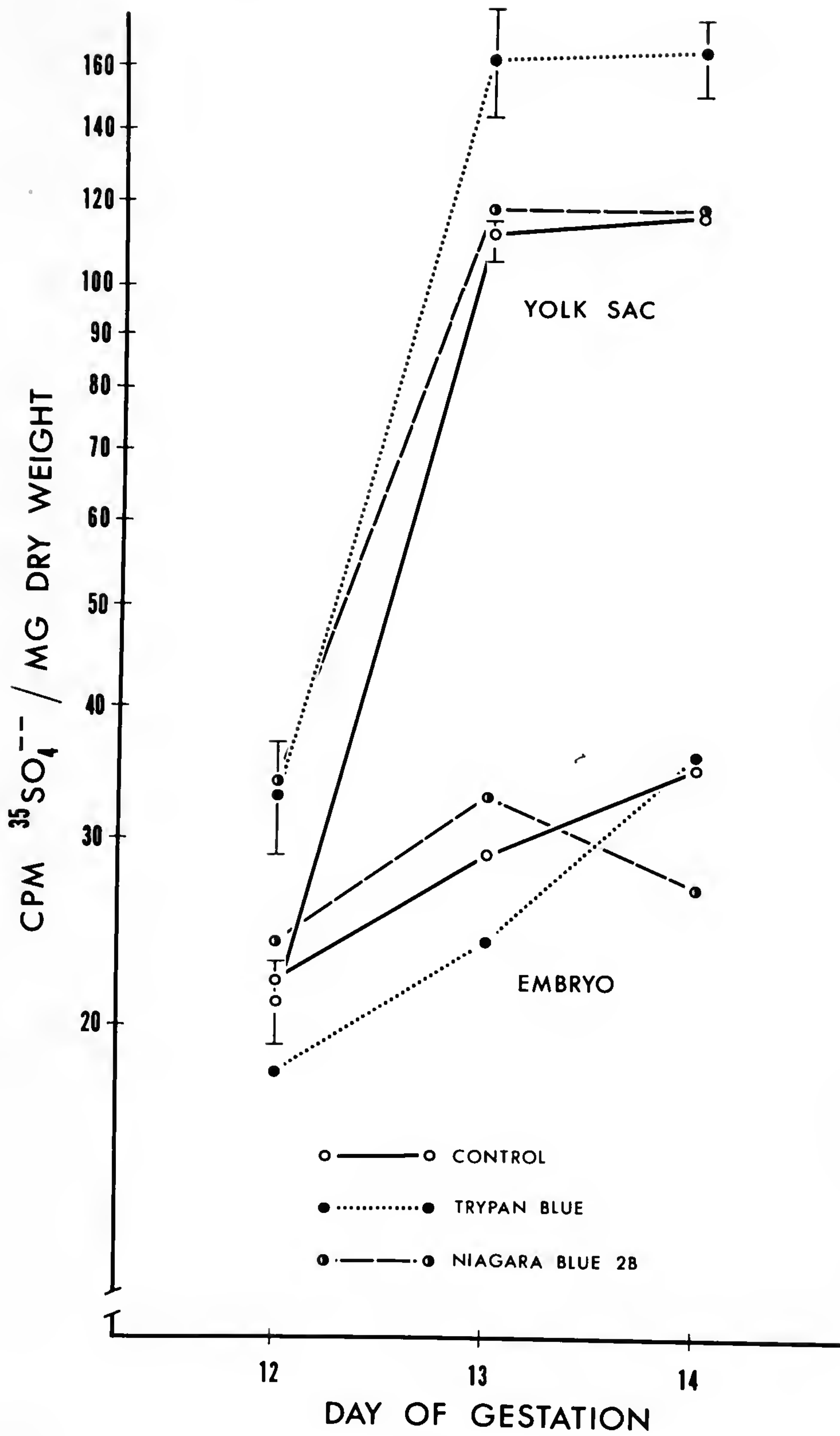

160
140
120
100
90
80
70
60
50
40
30
20
CPM $^{35}SO_4^{--}$ / MG DRY WEIGHT
YOLK SAC
EMBRYO
CONTROL
TRYPAN BLUE
NIAGARA BLUE 2B
12
13
14
DAY OF GESTATION

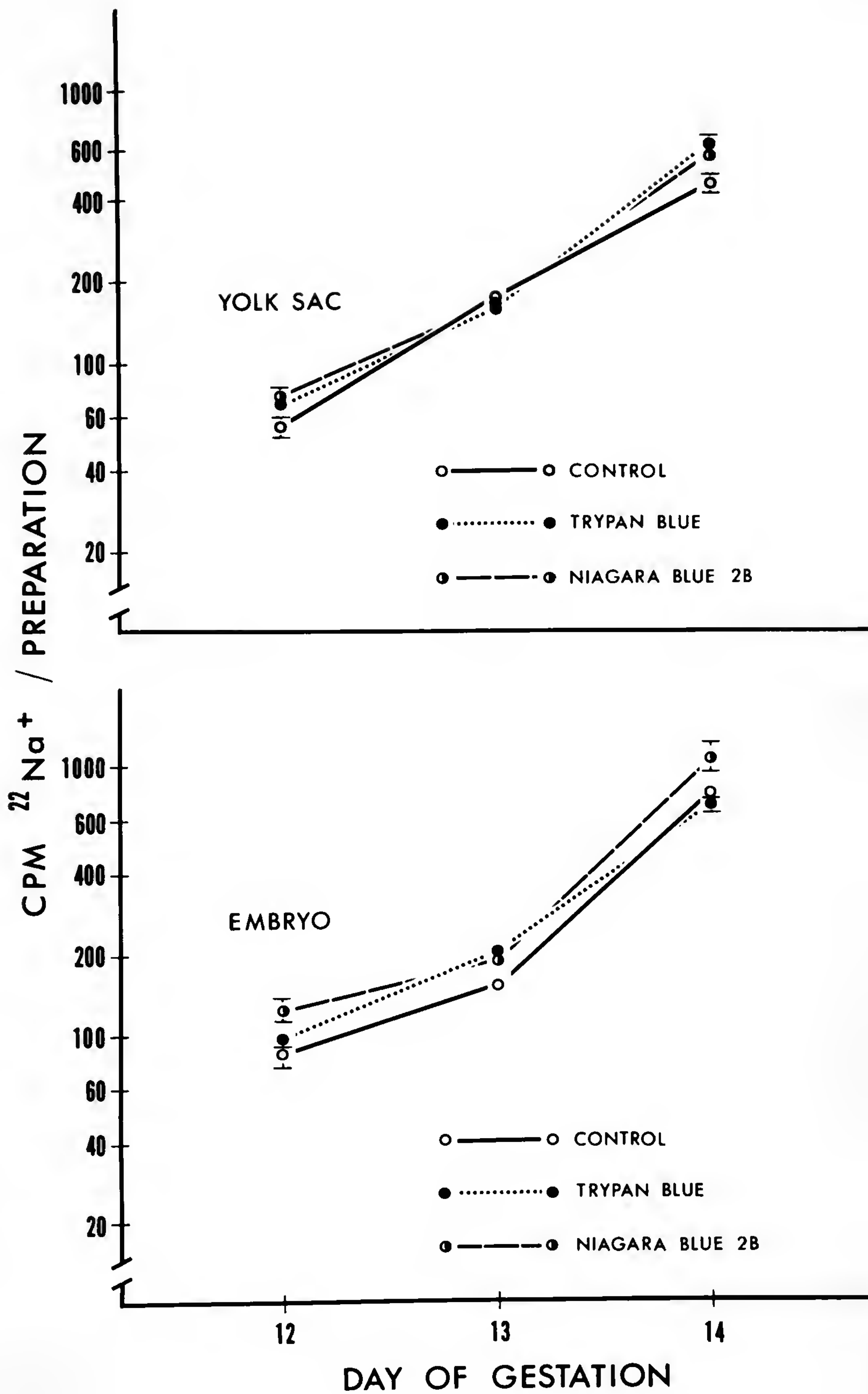
TABLE 5

ABSORPTION OF $^{22}Na^{+}$ [a]

	CPM $^{22}Na^{+}$/Preparation			CPM $^{22}Na^{+}$/mg Dry Weight		
	Control	Trypan Blue	Niagara Blue 2B	Control	Trypan Blue	Niagara Blue 2B
Day 12						
Yolk-Sac	58 ± 4 (22/4)	71 ± 5 (26/5)	73 ± 5 (23/4)	81 ± 5 (22/4)	105 ± 7 (26/5)	116 ± 8 (23/4)
Embryo	85 ± 9 (21/4)	95 ± 7 (26/5)	122 ± 13 (23/4)	29 ± 3 (21/4)	35 ± 3 (26/5)	43 ± 5 (23/4)
Day 13						
Yolk-Sac	171 ± 9 (24/4)	162 ± 10 (19/4)	167 ± 9 (18/3)	95 ± 6 (24/4)	105 ± 8 (19/4)	102 ± 6 (18/3)
Embryo	153 ± 13 (24/4)	199 ± 43 (18/4)	189 ± 21 (18/3)	22 ± 2 (24/4)	30 ± 5 (18/4)	33 ± 4 (18/3)
Day 14						
Yolk-Sac	462 ± 33 (18/3)	633 ± 55 (18/4)	570 ± 39 (16/3)	144 ± 10 (18/3)	219 ± 17 (18/4)	186 ± 14 (16/3)
Embryo	785 ± 141 (18/3)	703 ± 48 (18/4)	1040 ± 45 (16/3)	58 ± 10 (18/3)	55 ± 4 (18/4)	80 ± 11 (16/3)

[a] See text and Table 1 for details of treatment. Data expressed as mean ± standard error. Figures in parentheses indicate: (number of preparations/number of mothers).

Fig. 11.—Semi-logarithmic graph comparing mean tissue absorption of $^{22}Na^{+}$ with gestational age. Vertical bars represent standard errors and are only present when a significant difference ($p < 0.05$) exists. See Table 5 for numerical data.

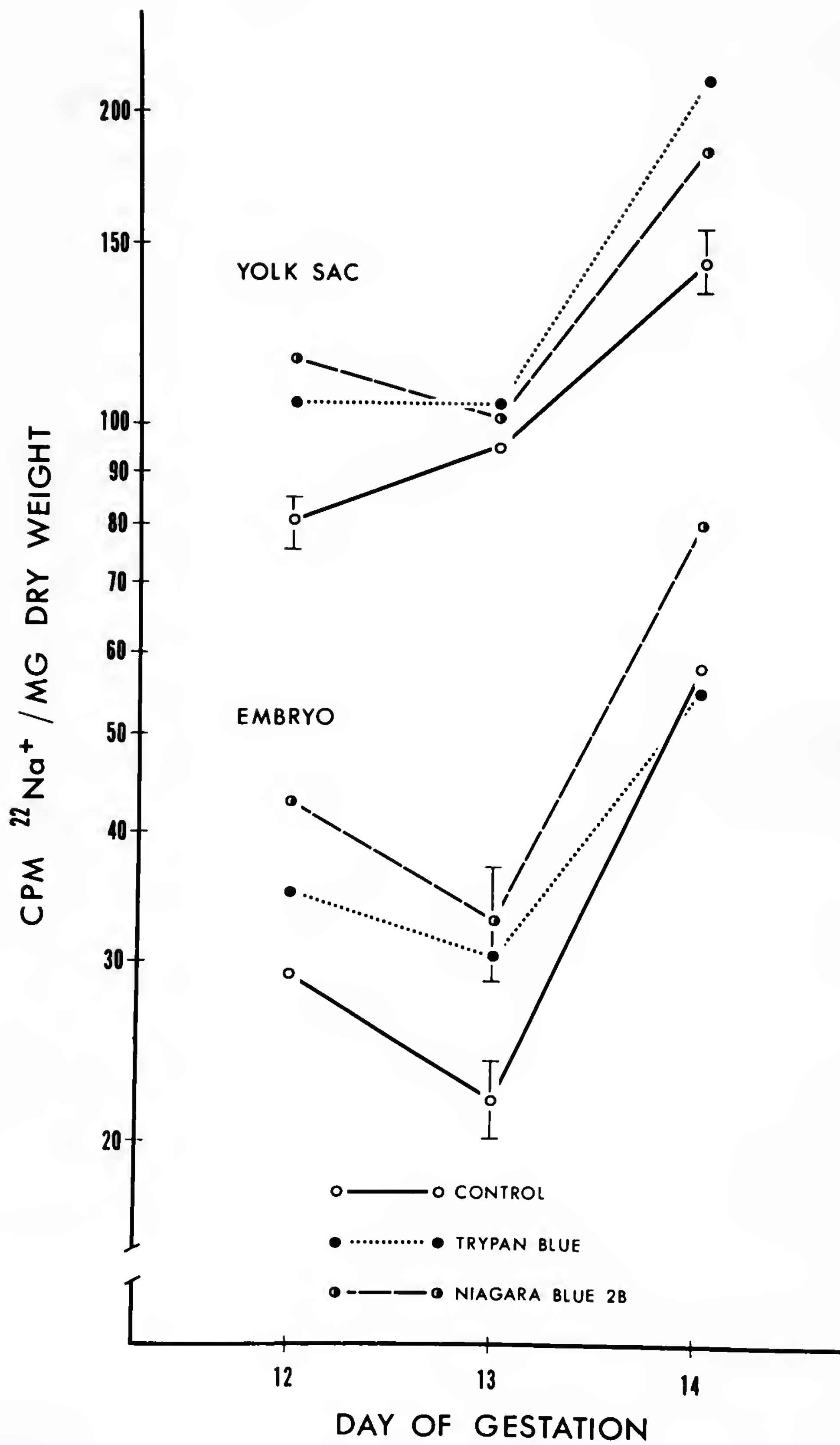

CPM $^{22}Na^{+}$ / PREPARATION
YOLK SAC
1000
600
400
200
100
60
40
20
CONTROL
TRYPAN BLUE
NIAGARA BLUE 2B
EMBRYO
1000
600
400
200
100
60
40
20
CONTROL
TRYPAN BLUE
NIAGARA BLUE 2B
12
13
14
DAY OF GESTATION

yolk-sacs stained with Niagara blue 2B accumulate a significantly greater amount of label than the controls, but then seem to recover such that there are no differences at days 13 and 14. The presence of trypan blue in the vitelline epithelium apparently results in a significant increase in incorporation over control levels only at day 14. Prior to that time, trypan blue-treated and control values are not different and no significant differences between yolk-sacs treated with trypan blue or Niagara blue 2B are apparent.

The embryos also seem to increase their uptake of $^{22}Na^{+}$ with gestational age, though the rate of increase between days 12 and 13 is less than between days 13 and 14. Day 12 embryos from Niagara blue 2B-treated females absorb a greater amount of label than the controls, while control values at all other stages are not different from either of the experimental groups. However, at day 14, Niagara blue 2B-treated embryos absorb a significantly greater ($p < 0.05$) amount of label than those embryos exposed to trypan blue.

For each group tested, the specific activities of yolk-sacs and embryos seem to parallel one another at each day of gestation (Fig. 12). Both trypan blue- and Niagara blue 2B-treated yolk-sacs have significantly greater $^{22}Na^{+}$ specific activities than control yolk-sacs at days 12 and 14, but are not different from one another. At day 13, no differences between any of the groups are apparent. The specific activities of the embryos are similar and the only statistically significant differences which occur are on day 13, when the Niagara blue 2B treatment results in a greater specific activity than the control.

Fig. 12.—Semi-logarithmic graph comparing mean tissue $^{22}Na^{+}$ specific activity with gestational age. Vertical bars represent standard errors and are only present when a significant difference ($p < 0.05$) exists. See Table 5 for numerical data.



YOLK SAC
EMBRYO
CPM $^{22}Na^{+}$ / MG DRY WEIGHT
200
150
100
90
80
70
60
50
40
30
20
CONTROL
TRYPAN BLUE
NIAGARA BLUE 2B
12
13
14
DAY OF GESTATION

Effects of Trypan Blue on the in Vivo Uptake of $^{35}SO_4^{--}$

Autoradiographs were prepared to determine if the in vitro changes in $^{35}SO_4^{--}$ uptake reflect similar in vivo changes or if they merely result from the artificial conditions employed. Table 6 is a summary of the mean grain counts over the chorio-allantoic placenta, visceral yolk-sac and embryo at 1 and 3 hours after injecting pregnant females on day 13 of gestation with the isotope. The number of counts over all tissues studied soon after injection (15 and 30 minutes) were not significantly greater than background and are not reported.

All grain counts were taken in the following manner. A grid containing 49 squares was placed in a 10X wide-field ocular. Under oil immersion, the number of developed grains were counted over several fields from several sections taken from 2 embryos from each pregnant female. Only those grains in the 13 squares forming an X in the center of the grid were counted. An exception was the yolk-sac villi where the whole villus was counted. The means for the chorio-allantoic placenta were calculated on the basis of the grain counts over 3 fields each of the metrial gland, decidua basalis and junctional zone. Background (the mean of 4 fields counted in the emulsion around each section studied) was subtracted from each count and the sum of the counts was divided by the number of fields. The mean grain count for the visceral yolk-sac was calculated on the basis of fields over 8 villi and 2 fields over the nonvillous region, while the mean number of counts over the embryo included fields counted over the neural tube, limb bud, notochord and loose mesenchyme.

TABLE 6

IN VIVO $^{35}SO_4^{--}$ INCORPORATION[a]

Tissue	Control Grain Count	Trypan Blue Grain Count	Per Cent of Control
One Hour			
Chorio-Allantoic Placenta	70	196	280
Visceral Yolk-Sac	30	51	170
Embryo	61	193	316
Three Hours			
Chorio-Allantoic Placenta	433	381	88
Visceral Yolk-Sac	178	189	106
Embryo	202	273	135

[a]The mean counts presented in this table have been corrected for background. See text for further details.

By 1 hour after injection of the label, the trypan blue-treated tissue had absorbed 170 to 316 per cent more $^{35}SO_4^{--}$ than the control, with the largest percentage of increase being found in the amount reaching the embryo. After 3 hours, the ratio of counts between trypan blue-treated and control tissues had decreased so that the number of grains over the control and experimental placentae (Fig. 13) and yolk-sacs (Fig. 14) was essentially identical. The abnormally developing embryos (Fig. 15), however, seem to absorb more than the controls. Although the data indicate that trypan blue treatment results in a greater uptake of $^{35}SO_4^{--}$, the significance of these increases is not assured until information from the progeny of more than 1 pregnant female is included.

Fig. 13.—Autoradiographs of the junctional zone of the chorio-allantoic placenta. Stained with hematoxylin and eosin. 800X.

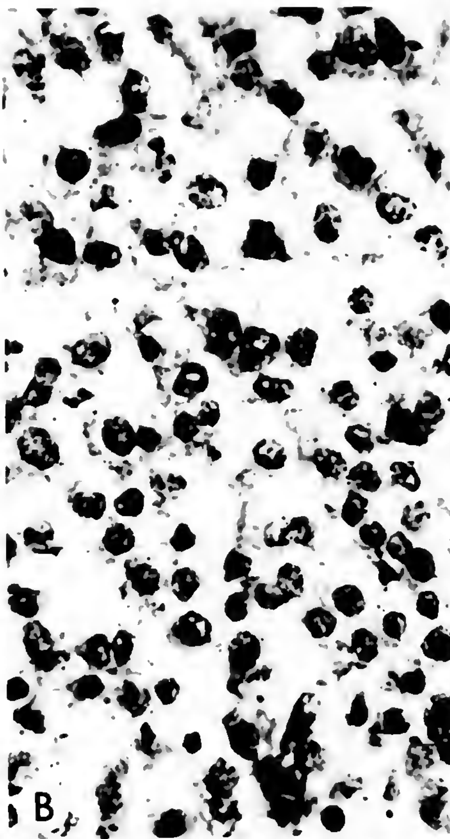
A. Normal control, 1 hour after injection of label.

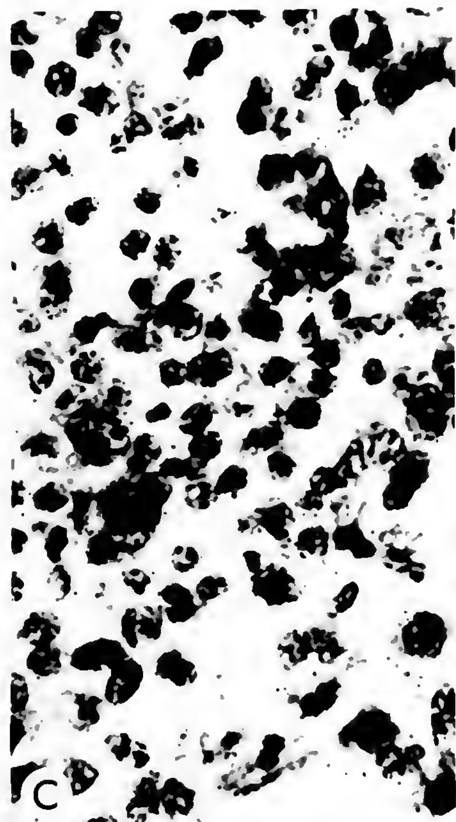
B. Trypan blue-treated, 1 hour after injection of label.

C. Normal control, 3 hours after injection of label.

D. Trypan blue-treated, 3 hours after injection of label.

A


B


C

D

Fig. 14.—Autoradiographs of villi from the visceral yolk-sac. Stained with hematoxylin and eosin. 800X.

A. Normal control, 1 hour after injection of label.

B. Trypan blue-treated, 1 hour after injection of label.

C. Normal control, 3 hours after injection of label.

D. Trypan blue-treated, 3 hours after injection of label.

A B

C D

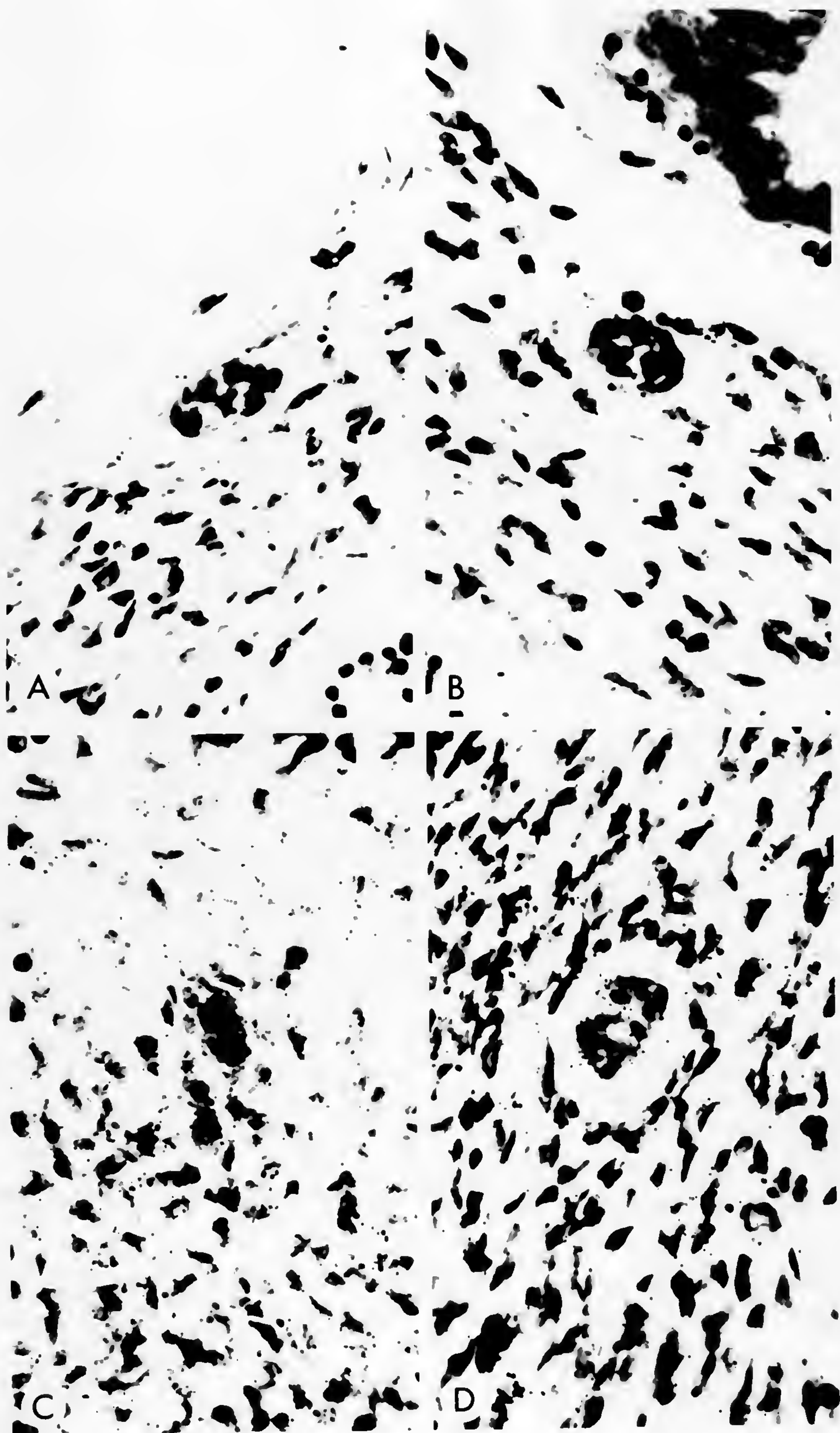
Fig. 15.—Autoradiographs of embryonic mesenchyme in the region of the notochord. Stained with hematoxylin and eosin. 800X.

A. Normal control, 1 hour after injection of label.

B. Trypan blue-treated, 1 hour after injection of label.

C. Normal control, 3 hours after injection of label.

D. Trypan blue-treated, 3 hours after injection of label.


A
B
C
D

DISCUSSION

Incidence of Gross Malformation

A review of the literature concerning experiments with trypan blue and Niagara blue 2B-induced congenital malformations in rats reveals much variation from investigation to investigation in the incidence of malformation. In general, this variation depends upon the strain of rats studied; the time, dose and route of administration and, probably of even greater consequence, the purity of the dye. Under the conditions of the experiments presented in this dissertation (see Table 7 and Materials and Methods), a 49 - 64 per cent incidence of malformation in surviving fetuses was noted. Each of these fetuses exhibited at least one abnormality resulting from treatment with trypan blue. The dye also caused a high rate of embryonic death as indicated by the percentage of resorbing implantation sites.

On the other hand, Niagara blue 2B, an azo dye of quite similar molecular structure and colloidal property (Wilson *et al.*, 1959), appears to be a relatively impotent teratogen; treatment with it resulting in a low malformation rate and a resorption rate of less than one-half that of trypan blue. Although Niagara blue 2B is absorbed by the vitelline epithelium and maternal reticulo-endothelial system to much the same extent as trypan blue (Wilson, *et al.*, 1959), it does differ in other physiological properties. For example, Niagara blue 2B is more toxic to the mother even though it apparently has a shorter serum half-life (Lloyd and Beck, 1966).

TABLE 7

INCIDENCE OF TRYPAN BLUE- AND OF NIAGARA BLUE 2B-INDUCED CONGENITAL MALFORMATION IN THE RAT

Azo Dye	Author and Year	Strain of Rat	Number of Females	Total Dose and Route of Administration	Day Injected	Number of Sites	Per Cent Re-sorbed	Per Cent Survivors Malformed
Trypan Blue	Wilson, 1955	Wistar descendant	45	120-150 mg/kg subcutaneous	7, 8, 9	407	44	49
	Beaudoin and Pickering, 1960	Sherman	19	140 mg/kg intraperitoneal	8	181	57	54
	Beaudoin and Kahkonen, 1963	Wistar	19	140 mg/kg intraperitoneal	8	231	54	64
	Kernis (this dissertation)	Long-Evans	22	167 mg/kg subcutaneous	8	240	46	62
Niagara Blue 2B	Wilson, _et al._, 1959	Wistar descendant	11	120-150 mg/kg subcutaneous	7, 8, 9	116	7	0
	Beaudoin, 1962	Sherman	16	140 mg/kg intraperitoneal	8	165	24	0
	Lloyd and Beck, 1966	Wistar	12	150 mg/kg subcutaneous	8.5	128	41	39
	Kernis (this dissertation)	Long-Evans	12	167 mg/kg subcutaneous	8	144	7	2

The results of the experiments reported in this dissertation (Table 1) confirm the previously reported investigations with respect to incidence of malformation and resorption. In addition, these data indicate that at least 3 out of 5 of the trypan blue-treated preparations incubated *in vitro* for the ion uptake studies were destined to have at least one abnormality, while about 1 out of 50 Niagara blue 2B-treated embryos would be malformed.

The observation that the primary site of malformation was the central nervous system is not remarkable in the light of present theories. Presumably, damage to a developing structure or system occurs only at that time in differentiation which is critical to the normal biochemical or morphological development of that system (Kalter and Warkany, 1959). Consequently, the central nervous system, including the special sense organs, which is undergoing its biochemical and morphological differentiation at day 8, is affected most severely by treatment at that day. The appearance of abnormalities of other organ systems may be explained by postulating residual effects of the dye which result in alterations of structures developing during a later stage in gestation. The fact that both trypan blue and Niagara blue 2B are present in the maternal tissues and visceral yolk-sac for an extended period of time would tend to support the concept of residual effectiveness.

But what exactly are these effects? Gillman *et al*. (1948) showed that trypan blue was absorbed in apparently high quantities by the phagocytes of the maternal reticulo-endothelial system. The fact that some nonteratogenic azo dyes were not phagocytized by this system (Wilson, 1955) caused Wilson *et al*. (1959) to study whether or not only

teratogens were taken up by the phagocytes. Instead, they found both Niagara blue 2B and India ink to be actively absorbed by the maternal reticulo-endothelial system. It was therefore concluded that teratogenesis is not influenced by a loading of this system.

Gillman et al. (1948) also demonstrated that trypan blue is bound to the albumin fraction of maternal plasma protein, while Beaudoin and Kahkonen (1963) showed a decrease in total fetal protein concentration as well as decreases in beta globulin, alpha-1-globulin and albumin concentrations at day 20, after previous maternal injection with trypan blue. No such information is available from studies with Niagara blue 2B-treated pregnant rats. Whether these changes are directly concerned with teratogenesis, either as causes or effects, remains to be determined.

As a matter of fact, it is still not clear whether the embryo, the maternal organism or the visceral yolk-sac is the direct site for trypan blue action. Since the dye has never been seen to penetrate the rat embryo and since it is a potent teratogen in chicks which would indicate that no maternal influence is involved, Beck et al. (1967) have concluded that the most reasonable site of action for trypan blue in the rat is the visceral yolk-sac. Indeed, under in vitro circumstances, they have demonstrated that increasing concentrations of trypan blue are able to inhibit the activities of certain enzymes isolated from the lysosomes of near-term visceral yolk-sacs. Accordingly, they suggest that the inhibition of those lysosomal enzymes, i.e., beta-glucuronidase, acid phosphatase, ribonuclease and deoxyribonuclease, results in the inability on the part of the visceral endoderm to digest absorbed

material. Any possible barrier to these large undigested molecules would result in a lack of transfer of nutritional elements to the embryo. Although this theory is quite attractive, judgment should be reserved until it is substantially shown that (1) trypan blue does indeed penetrate into the lysosomes, (2) the enzyme inhibition occurs *in vivo* and (3) such an inhibition also occurs in yolk-sacs from earlier stages in gestation, particularly at that critical time in development when trypan blue is most effective in producing malformations.

In Vitro Ion Uptake

Since the available evidence indicated that trypan blue might have an effect on yolk-sac function, it became desirable to test this hypothesis in terms of the organ's ability to absorb ions under *in vitro* and *in vivo* conditions.

The explanted embryo-in-yolk-sac preparations utilized for these experiments appear to sustain themselves quite well in the culture medium. This was indicated by heart beat and yolk-sac perfusion. In addition, Netzloff *et al*. (1968) demonstrated that these preparations could consume oxygen linearly with time for at least 1½ hours. These observations suggest that the preparations were indeed viable. Therefore, the data presented in this dissertation were derived from robust, living tissues rather than from moribund or necrotic preparations.

Unlike the uptakes of vitamin B_{12} or vitamin B_{12}-intrinsic factor complex (Padykula *et al*., 1966), the specific activities of tagged ions do not seem to decrease with gestational age. Instead, there is a general increase with time and weight such that there is no

reduction of yolk-sac ion absorption between days 12 and 14. No information concerning ion uptake by this tissue at later stages of gestation is available. As a result, the suggestion that yolk-sac function is reduced as gestation proceeds (Padykula et al., 1966; Jollie, 1964) cannot be supported by these experiments.

With the exception of the $^{45}Ca^{++}$ specific activities of day 14 yolk-sacs (Fig. 8), wherever there is a significant difference between the trypan blue-treated and control yolk-sacs or embryos, the specific activity of the dye-treated tissue always is greater. Since day 12 and 13 trypan blue-treated yolk-sacs weigh significantly less than the corresponding controls, the increased specific activities indicate that either a smaller amount of protein or a fewer number of cells (or both) is capable of absorbing the same or greater amounts of ion. Although Niagara blue 2B treatment does not result in reduced tissue weights, where statistical differences do exist, the dye-treated tissues have greater specific activities than controls. This phenomenon would tend to suggest that the machinery used by the yolk-sac to absorb ions is altered by some interaction with Niagara blue 2B. This interaction is as yet unidentified.

Although the absorption of ions by embryos generally parallels the uptake by yolk-sacs as gestation proceeds, there appears to be no consistency in the relative amounts of ions taken up when the yolk-sacs are compared to similarly treated embryos at the same day of development. For example, consider Figure 9 which depicts the absorption of $^{35}SO_4^{--}$ on a preparation basis. On day 13, the trypan blue-treated yolk-sacs absorb a significantly greater amount of label than the corresponding

controls, while the 13-day Niagara blue 2B-treated embryos incorporate a significantly greater amount of label than the trypan blue-treated. A more striking example of this situation is presented in Figure 12. Statistical analysis demonstrates that at day 13, control and experimental yolk-sacs show no differences in their capacity to absorb $^{22}Na^{+}$. The day 13 embryos after Niagara blue 2B treatment, however, have a significantly greater $^{22}Na^{+}$ specific activity than the controls, but are not different from those treated with trypan blue.

These results suggest that the interrelationship between the embryo and its yolk-sac is very complex. The presence of trypan blue in the yolk-sac at day 13 increases the $^{35}SO_4^{--}$ uptake, but the increase is not reflected in the embryo. However, the presence of Niagara blue 2B on day 13, has no effect on the yolk-sac's ability to absorb sulfate, while it may cause an increase in the amount of ion passing into the embryo. With regard to $^{22}Na^{+}$, again no differences are seen in day 13 yolk-sacs, while treatment with Niagara blue 2B causes an increased amount of label to penetrate into the embryo. Since the presence of trypan blue in the yolk-sac was never correlated with a concomitant significant difference in the embryo, there is the possibility that the dye may prohibit the passage of these particular ions at these particular stages in development. Whether the same holds true for other stages of development and for other ions or organic molecules has not been determined.

The relationship between treated and control tissues may also change from day to day. For example, Niagara blue 2B-treated day 12 yolk-sacs (Fig. 9) absorb a significantly greater amount of sulfate

than the control, while at day 13 the presence of trypan blue causes an increase in absorption when compared to controls. Figure 10 also demonstrates these changing relationships. Trypan blue-treated yolk-sacs have significantly greater specific activities than the controls at each day of gestation. The day 12 Niagara blue 2B-treated yolk-sacs also absorb more sulfate than the controls. The change occurs at day 13 when there is no difference between Niagara blue 2B and controls, thus indicating a possible recovery. Conceivably, recovery from Niagara blue 2B treatment could occur more rapidly than from treatment with trypan blue, for the former is excreted from the maternal tissues and the proximal yolk-sac at a greater rate than the latter.

The fact that there are changes in the specific activities or amounts of different ions absorbed by control and experimental tissues on varying days of gestation is not at all surprising. Embryonic and yolk-sac tissues are undergoing a rapid and extensive biochemical and morphological differentiation during this period of gestation. As a result, it is quite likely that as the tissues differentiate, their ionic and nutritional requirements are altered with their particular needs at varying stages of development. More important, however, is that the presence of azo dyes can affect the ability of yolk-sacs to absorb and possibly transfer certain ions. For this reason, the complex relationship between the embryo and its yolk-sac with particular regard to the exchange of materials should be studied further. These kinds of experiments might indeed show that the yolk-sac plays an important role in normal embryonic differentiation and growth, so that alterations in normal yolk-sac function could be a mechanism of teratogenesis.

In Vivo Uptake of $^{35}SO_4^{--}$

The increased *in vitro* uptake of $^{35}SO_4^{--}$ by trypan blue-treated tissues when compared to controls was confirmed by autoradiographic procedures. Although the number of pregnant females utilized for this purpose was not sufficiently great to warrant statistical analyses, the results also confirm those seen by Kochhar *et al*. (1968). These investigators noted a significant, dose-dependent increase in the absorption of $^{35}SO_4^{--}$ by trypan blue-treated mouse embryos at days 10, 11 and 12 of gestation.

Since the maternal administration of $^{35}SO_4^{--}$ has been shown to result in a high uptake by fetal mesenchyme or mesenchyme derivatives (Boström and Odeblad, 1953) and since one of the end results of trypan blue treatment is a paucity of embryonic mesenchyme (Chepenik, 1965), it seems unlikely that less mesenchymal tissue is able to incorporate more ion. Indeed, Kochhar *et al*. (1968) found that the $^{35}SO_4^{--}$ was not incorporated into normal sulfated organic compounds, but, instead, was present in greater amounts as the inorganic sulfate ion or in compounds of low-molecular weight. The increase in sulfate ion shown in both the current and previous studies could very well indicate that the organ transferring material between mother and embryo is the affected organ and that, somehow, the presence of trypan blue results in an increased placental permeability to sulfate. Whether the primary effect is on the chorio-allantoic placenta or the proximal yolk-sac is still unclear.

CONCLUSIONS

1. When injected into pregnant rats on day 8 of gestation, trypan blue is a potent teratogen. It results in a high percentage of congenital malformations, primarily of the central nervous system and special sense organs. This effect is apparently due to the rapid biochemical and morphological differentiation which is taking place in these systems at the time of insult. Abnormalities of other systems conceivably result from a residual effectiveness suggested by the slow rate of excretion from the maternal organism.

2. Niagara blue 2B is considerably less effective as a teratogen, but still results in a rate of congenital malformation which is significantly greater than the spontaneous incidence of malformation for the Long-Evans black-hooded strain of rats. Its lower effectiveness could be the result of its more rapid rate of excretion.

3. On days 12, 13 and 14 of gestation, both dyes cause a significant increase in the absorption of $^{45}Ca^{++}$, $^{35}SO_4^{--}$ or $^{22}Na^{+}$ by yolk-sacs and, in some cases, abnormally developing embryos. These changes in the specific activities of yolk-sacs suggest that both dyes have an effect on normal yolk-sac function and indicate that alterations in the function of the yolk-sac can conceivably bear a direct relationship to the induction of congenital abnormalities. Future studies, however, must utilize a teratogen which is effective at later days of

gestation, so that transfer phenomena may be studied before, during and after the teratogenic insult.

4. Autoradiographic studies on day 13 control and trypan blue-treated implantation sites confirmed the results of the *in vitro* experiments. The preliminary data from teratogen-treated chorio-allantoic placentae, yolk-sacs and embryos indicate that all three tissue types absorb greater amounts of $^{35}SO_4^{--}$ than the corresponding controls.

5. It is proposed that the proximal yolk-sac is a functioning organ at this stage of gestation and that an alteration in the relationship between the embryo and its yolk-sac could be significant in the induction of congenital abnormalities.

APPENDICES

APPENDIX A

Composition of Bouin's Fluid

Saturated aqueous picric acid	75 parts by volume
Formalin (40% formaldehyde)	25 parts by volume
Concentrated glacial acetic acid	5 parts by volume

APPENDIX B

Composition of Phosphate-Ringer Buffer

Substance	millimoles/liter	grams/liter
Solution A		
NaCl	145	8.50
KCl	5.6	0.42
Na_2HPO_4	4.2	0.60
H_3PO_4	Add to pH 7.4	
$MgSO_4$	1.2	0.145
Solution B		
$CaCl_2$	2.16	0.24

Mix 8 parts A : 1 part B

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VITA

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He has accepted the position of Assistant Professor of Anatomy at the University of Illinois College of Medicine in Chicago.

He was married in August, 1966, to the former Michele Phyllis Hinden of Sarasota, Florida.

This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of that committee. It was submitted to the Dean of the College of Medicine and to the Graduate Council, and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

June, 1968

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